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(54) Title: ZOOCIN A IMMUNITY FACTOR (57) Abstract <p>The invention relates to a factor which has activity in protecting a cell against the bacteriolytic enzyme, zoocin A. Nucleic acid which encodes the factor is useful in transforming GRAS organisms to be able to produce zoocin A without vulnerability to the activity of the enzyme itself. The resulting organisms can then be used in antibacterial compositions (particularly foodstuffs) against a range of bacteria, including <i>S. mutans</i>, <i>S. sobrinus</i> and <i>S. pyogenes</i>.</p>		

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ZOOCIN A IMMUNITY FACTOR

TECHNICAL FIELD

- 5 The invention relates to a factor which has activity in protecting a cell producing zoocin A, to the gene encoding that factor, to vectors and organisms containing the gene and the use of such organisms as anti-bacterial agents.

BACKGROUND ART

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Since the dawn of microbiology it has been observed that the growth of some strains of bacteria can interfere with the growth of other potentially harmful bacteria growing in the same medium. We now know that these inhibitory reactions are mediated by a range of metabolic and protein products produced by many different strains of bacteria. The "classical" antibiotics such as streptomycin and penicillin are metabolic (enzyme synthesized) products and their use in the prevention and treatment of disease is now well established. In contrast, industrial and medical use of proteinaceous (ribosomally synthesized) inhibitory substances has been much more limited. Recently however, this situation has changed and in 1988 nisin was granted GRAS (Generally recognized as safe) status by the U.S. Food and Drug Administration (Federal Register 1988) in recognition of the fact that nisin was produced by *Lactococcus lactis* strains naturally associated with certain foods during processing and that it has no apparent adverse effects when ingested.

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25 Zoocin A is a unique domain-structured bacteriolytic enzyme produced by *Streptococcus equi* subsp. *zooepidemicus* 4881, which specifically attacks the cell walls of some closely related streptococcal species including the principal causative agents of group A streptococcal sore throat and dental caries respectively (Simmonds *et al* (1995); Simmonds *et al* (1996)). It was shown that zoocin A could suppress the growth of *S. mutans* in a triple species plaque model and that the initiation of the killing sequence occurred very quickly. A 6.8 kb *EcoR* I fragment containing the gene encoding zoocin A (*zooA*) was cloned into *Escherichia coli* using the pBluescript® II SK(+) phagemid vector and the sequence of *zooA* determined (Simmonds *et al* (1997)). The N-terminal catalytic domain of zoocin A has a high degree of homology with the N-terminal catalytic domain of a similar bacteriolytic

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enzyme lysostaphin, produced by *Staphylococcus simulans* biovar *staphylolyticus*, which specifically attacks the cell walls of other staphylococcal species. The C-terminal substrate-binding domain of lysostaphin is known to have a high degree of homology to at least one other staphylococcal cell wall binding enzyme, a *Staph. aureus* amidase. By contrast, the substrate-binding domain of zoocin A has homology to no other known sequence. Both enzymes appear to lyse cell walls by cleaving the peptide cross-links within the peptidoglycan (Simmonds *et al* (1996)). The bacteriocidal nature of their mode of action and the high degree of species and strain specificity exhibited by these enzymes are characteristics of that group of proteinaceous inhibitory agents referred to as bacteriocin-like inhibitory substances (BLIS).

Zoocin A targets only a very limited range of bacteria, restricted to some species of *Streptococcus* only. This species-specific anti-bacterial action is useful. For example, it is active against two groups of medically significant human pathogens and at least one significant animal pathogen.

S. mutans and *S. sobrinus* are two of twenty or more species of bacteria present in dental plaque. Although not numerically dominant, these two species are considered to be the major aetiological agents of dental caries and their suppression in the oral cavity has been shown to reduce caries incidence (Loesche (1976); Loesche *et al* (1989)). Group A streptococci (GAS) infect via the upper respiratory tract where the tonsillar region in particular is believed to be the primary site of colonization. GAS carriage in humans is relatively common and GAS pharyngitis left untreated can progress to more serious disease including rheumatic fever and nephritis (Bronze and Dale (1996)). Vaccines are not available to prevent these infections and although it has been shown that these groups of microorganisms can be suppressed in the oral cavity by administration of antibacterial agents such as chlorhexidine (Loesche (1976)), polyvalent cations (Jones *et al* (1988)) and classical antibiotics (Loesche *et al* (1989)), the broad spectrum nature of these agents means that many commensal organisms are also suppressed, a condition which is known to pre-dispose the patient to superinfection by resistant microorganisms including gram-negative bacteria and yeasts. In each case the prolonged and widespread use of these agents has not been considered acceptable (Marsh (1991)). In contrast, zoocin A, while having significant bacteriocidal activity against these groups of

microorganisms has little or no activity against many other plaque species such as *S. oralis* (Simmonds *et al* (1996)), *S. sanguis* or non-streptococcal species (Simmonds *et al* (1995)), or against the major groups colonizing the mucosal surfaces of the oral cavity such as *S. salivarius* (Simmonds *et al* (1995)). Therefore, administration of zoocin A to the oral cavity is unlikely to result in the complications seen with the previously mentioned broad spectrum anti-microbial agents, yet should lead to a decrease in the incidence of dental caries and carriage of GAS.

Before zoocin A can be used for its desirable anti-bacterial properties, there is a need for it to be provided in a form that can be administered to a human or an animal safely. For many antibiotics this is achieved by batch fermentation of the organism producing the antibiotic and purifying the antibiotic molecule and adding it to a suitable carrier. This method would be very expensive for zoocin A which has a molecular weight of 28,000. For that reason, the more commercially attractive option is to produce the zoocin A *in situ* in a naturally fermented food such as yoghurt.

However, zoocin A is produced by *S. equi* subsp. *zooepidemicus*, a recognized animal and occasional human pathogen. Serious human disease has been shown to result from the ingestion of *S. equi* subsp. *zooepidemicus* contaminated unpasteurized milk (Francis *et al* (1993)). Therefore, use of the natural producer organism to incorporate zoocin A in a food product as part of a food fermentation process is unlikely to be acceptable, but one solution would be to move the genes required for zoocin A production from the natural host to an organism suitable for use in food fermentation processes. However, this approach presents some difficulties when zoocin A is lethal to the genetically transformed organism.

One solution to these difficulties is to render the organism which is to express zoocin A resistant (immune) to the activity of this enzyme. This solution requires a factor to be identified which protects otherwise susceptible organisms against zoocin A activity.

The applicants have now identified such a factor, which is generally referred to hereinafter as zoocin A immunity factor. It is towards this factor and to its use that the present invention is broadly directed.

SUMMARY OF THE INVENTION

5 In one aspect, the invention provides zoocin A immunity factor, which is a protein which is capable of protecting a host cell expressing zoocin A against the potentially damaging activity of zoocin A.

10 In a further aspect the invention provides an isolated DNA molecule which has a nucleotide sequence which encodes zoocin A immunity factor (*zif*).

15 Preferably the DNA molecule is selected from the group comprising molecules having one or more of: the *zif* sequence shown in Figure 3, a sequence comprising that sequence, a sequence comprising a part of that sequence active in protecting an organism from zoocin A, a sequence encoding the same protein as the *zif* sequence of Figure 3 but differing in nucleic acid sequence by virtue of degeneracy of the genetic code and a sequence which is a functionally equivalent variant of the *zif* sequence shown in Figure 3.

20 In still a further aspect of the invention, there is provided a vector comprising the *zif* encoding molecule defined above, optionally together with a gene encoding the zoocin A active protein or variant defined above.

25 In yet a further aspect, the invention provides a non-pathogenic organism containing the *zif* encoding molecule defined above, optionally together with a gene encoding a polypeptide sequence selected from the sequence for zoocin A or a functionally equivalent variant of that sequence.

Preferably, the organism is a food-grade organism.

30 As another aspect of the invention, there is provided an antibacterial composition comprising a non-pathogenic organism as defined above.

Preferably, the composition is suitable for ingestion, particularly human ingestion, and is a foodstuff, nutraceutical or confectionery.

In yet a further aspect, the invention provides a method of preventing or inhibiting the growth of undesirable organisms susceptible to zoocin A which comprises the step of contacting said organisms or the environment thereof with a composition as defined above.

Preferably, the organisms inhibited are *S. mutans*, *S. sobrinus* or *S. pyogenes* and the composition is administered to the oral cavity of a patient.

Other aspects of the invention will be apparent from the description provided, and from the claims.

DESCRIPTION OF THE DRAWINGS

While the invention is broadly as defined above, it further includes embodiments of which the following description provides examples. It will also be better understood with reference to the accompanying drawings in which:

Figure 1 shows a map of pBluescript® II SK(+) phagemid vector and pVA838.

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Figure 2 is a restriction map of PDN488L showing ORFs and subclones. The nucleotides are numbered from the first nucleotide of the *EcoR* I restriction site located proximal to the *Sac* I restriction site in the pBluescript® II SK(+) phagemid vector *Sac* I - *Kpn* I MCS of pDN488L. The translation is in the direction indicated by the bold arrows.

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Figure 3 shows the DNA sequence of 6.8 kb base *EcoR* I fragment showing the nucleotide and amino acid sequences for both *zooA* and *zif*. It will be appreciated that the strand of nucleic acid coding for *zif* is complementary to the non-coding strand shown expressly in Figure 3.

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DESCRIPTION OF THE INVENTION

The focus of the invention is on the applicants identification of the gene encoding zoocin A immunity factor (*zif*). This gene is capable of protecting cells which express
5 zoocin A against the effects of that enzyme.

The *zif* gene has been identified from *S. equi subsp. zooepidemicus* 4881 and has the sequence given in Figure 3. This sequence is of the non-coding strand, with the coding strand being complementary. The sequence of the coding strand is recited
10 as SEQ ID NO. 2.

However, it will be appreciated that the sequence need not always be that shown in Figure 3 but can instead be a functionally-equivalent variant of that sequence. Such variants are in no way intended to be excluded and the resultant molecules
15 are referred to herein as "*zif*-like genes".

The amino acid sequence of *zif* (which is coded for by the nucleotides of the coding strand) is also shown in Figure 3. Again, variations are possible while retaining functional equivalency.
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The phrase "functionally equivalent variants" recognises that it is possible to vary the amino acid/nucleotide sequence of a protein while retaining substantially equivalent functionality. For example, a protein can be considered a functional equivalent of another protein for a specific function if the equivalent protein is
25 immunologically cross-reactive with and has at least substantially the same function as, the original protein. The equivalent can be, for example, a fragment of the protein, a fusion of the protein with another protein or carrier, or a fusion of a fragment which additional amino acids. For example, it is possible to substitute amino acids in a sequence with equivalent amino acids using conventional
30 techniques. Groups of amino acids normally held to be equivalent are:

- (a) Ala, Ser, Thr, Pro, Gly;
- (b) Asn, Asp, Glu, Gln;
- (c) His, Arg, Lys;
- (d) Met, Leu, Ile, Val; and

(e) Phe, Tyr, Trp.

Equally, DNA sequences encoding a particular produce can vary significantly simply due to the degeneracy of the nucleic acid code.

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The probability of one sequence being functionally equivalent to another can be measured by the computer algorithms BLASTP (Altschul, S. F. *et al* (1990)) and FASTA (Pearson, W. R. *et al* (1988)) for proteins and DNA respectively.

10 The *zif* gene or *zif*-like gene of the invention can be inserted into organisms which are to be transformed with the *zooA* gene (which encodes zoocin A) so that a recipient organism which is zoocin A sensitive is protected by expression of the *zif* gene. The action of *zif* in protecting a zoocin A producer cell from the otherwise lethal action of its own product is believed to involve the modification of the cells
15 peptidoglycan cross-links to a chemical form non-hydrolysed by zoocin A.

Organisms which may be usefully transformed with the *zif* gene include any food-acceptable or pharmaceutically acceptable non-pathogenic organism. When the gene is inserted into zoocin A susceptible organisms, these organisms can be
20 subsequently or simultaneously transformed with *zooA* in a manner which allows production of zoocin A. The *zif* gene protects the transformed organism from the lethal effects of zoocin A produced.

It will of course be appreciated that the terms "transformed" or "transformation" are
25 used herein in their broadest possible sense. While normally a recombinant transformation process will be employed, any so-called "natural transfer" approach can also be used. "Natural transfer" approaches involve the placement of an organism including DNA encoding both *zif* and zoocin A in the proximity of the organism to which the DNA is to be transferred, and allowing exchange to occur
30 naturally.

Both recombinant and natural transfer of DNA from one host organism to another is now routine in the art. It will therefore be appreciated that any conventional approach can be employed, so long as the desired transformation occurs.

It will however be more usual to effect transformation by recombinant means. This is the preferred approach taken for this invention and normally will involve the use of transformation vectors/gene constructs.

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While it is conceivable that separate vectors/constructs could be employed to separately transfer the *zif* and zoo A genes to a recipient organism, it would be more usual for both genes to be contained in the same vector/construct.

10 The vector pSB1131 is a preferred vector for this purpose.

Preferred non-pathogenic organisms for use in the invention include yeasts and bacteria. In particular, organisms having a genus selected from non-pathogenic strains of streptococcus are particularly useful. Especially preferred are non-
15 pathogenic strains of *Streptococcus gordonii*.

Organisms transformed with the gene of the invention may be used as preservatives in processed cheese, various pasteurised dairy products, canned vegetables, hot baked flour products and pasteurised liquid egg. They may also be used in
20 preservation of naturally fermented foods such as beer, wine, yoghurt and cheeses.

The transformed organisms and/or extracts of the organisms may also be used to prepare pharmaceutical compositions for use topically to prevent establishment of infectious diseases of humans and animals. Such topical compositions are useful in
25 treatment of skin conditions, such as ulcers, in which streptococci are significant pathogens and where poor blood supply limits the effectiveness of systemically administered antibiotics.

Group C streptococci are serious animal pathogens, particularly of horses and are
30 responsible for considerable economic loss to the bloodstock industry. As with GAS in humans, the primary route of infection for these organisms is believed to be the respiratory tract and it is contemplated that the incorporation of organisms according to the invention which express zoocin A with animal feeds may reduce colonization rates in these animals, and hence the rate of serious disease.

It is however presently preferred that the transformed organisms and/or their zoocin A-containing culture fluid be included in a composition intended for human ingestion (such as a foodstuff, nutraceutical or confectionery). This is particularly the case where the intention is to treat or prevent problems associated with the organisms *S. mutans* and/or *S. sobrinus*. These organisms inhabit the oral cavity and, as stated previously, are considered to be the major aetiological agents of dental caries. Their suppression in the oral cavity reduces the incidence of dental caries.

Further, this is particularly the case where the intention is to treat or prevent problems associated with *S. pyogenes*. These organisms colonise the tonsillar region of the oral cavity and, as stated previously, are the major aetiological agents of GAS associated disease.

Foodstuffs such as processed cheeses and yoghurts are particularly appropriate for such applications. Confectioneries such as wine gums and chewing gums are also contemplated.

The transformed organism of the invention may be admixed with food products, confectioneries and pharmaceutical carriers by conventional means. For fermented products such as yoghurts, conventional methods may also be used including the step of adding the transformed microorganism at the time of culturing the product. Preferably the transformed microorganism is of the same species as conventionally used for the preparation of the fermented product thus allowing the preparation of the zoocin A and the fermented product to occur simultaneously.

The invention will now be described with reference to the following non-limiting examples.

EXAMPLE 1**Materials and Methods.****i) Bacterial strains and plasmids.**

5 Stock cultures of all strains were stored in skim milk at -70°C. Strains in regular use were maintained as plate cultures and subcultured every two weeks. *E. coli* DH5αF' (Woodcock *et al* (1989), Raleigh *et al* (1989)) was grown routinely at 37°C in air and *S. equi* subsp. *zooepidemicus* 4881 (Schofield and Tagg (1983)) and *S.gordonii* DL1 (Macrina *et al* (1982)) in 5% CO₂ in air atmosphere at 37°C.

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E. coli DH5αF' was routinely cultured in 2xYT medium (16 g bacto-tryptone (Difco Laboratories, Detroit, MI, USA), 10 g bacto-yeast extract (Difco), and 5 g NaCl (Riedel-de Haën AG, Seeize, Germany) to one litre of distilled water, purified with a Milli-Q system (Millipore Inc., France) (MQ water), Luria-Bertani (LB) medium (10 g bacto-tryptone (Difco), 5 g bacto-yeast extract (Difco), and 10 g NaCl (Riedel-de Haën AG) to one litre of MQ water) or on LB agar (LBA) plates. LBA was prepared by supplementing LB medium with 1.5% bacto-agar (Difco). Plates containing antibiotics were prepared by supplementing LBA with either 100 mg/ml ampicillin (LBA+Ap), 250 mg/ml erythromycin (LBA+Em250), 500 mg/ml erythromycin (LBA+Em500) or 25 mg/ml chloramphenicol (LBA+Cm). All antibiotics were manufactured by Sigma (Sigma Chemical Co., St. Louis, MO, USA). LBA containing antibiotics was stored at 4°C for periods of up to two weeks.

25 *Streptococcus gordonii* DL1 strains were routinely cultured in Todd Hewitt broth (THB) (Difco), on Columbia Agar Base (CAB) (GIBCO BRL, Life Tec. Ltd., Paisly UK) plates or on blood agar (BA) (CAB supplemented with 5% whole human blood (Dunedin Public Hospital, Dunedin, NZ)). Antibiotic containing agar plates were prepared by supplementing CAB with 10 mg/ml erythromycin (CAB+Em). Prior to transformation *S. gordonii* DL1 were grown in Brain Heart Infusion (BHI) (Difco) supplemented with 0.5% bacto-yeast extract (Difco), 1% membrane filtered horse serum (GIBCO BRL) and 0.1% glucose (Serva Feinbiochemica GmbH & Co. KG, Heidelberg, Germany) (BHS broth). CAB containing antibiotics was stored at 4°C for periods of up to two weeks.

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Bacterial strains and their plasmids used in this study are described in Table 1. Maps of pBluescript® II SK(+) phagemid vector (Stratagene, La Jolla, CA, USA) and pVA838 (Macrina *et al* (1982)) are given in Figure 1.

5 **ii) Genetic manipulations.**

Restriction enzyme digestion, ligation, and electrophoresis procedures.

Unless otherwise stated, cloning methods were carried out as previously described (Sambrook *et al* (1989)). Restriction digests were performed according to the manufacturers instructions; *EcoR* I, *Pst* I, *Hind* III, *Xba* I and *Pvu* II (Boehringer
10 Mannheim GmbH, Mannheim, Germany); *Cla*I and *EcoRV* (Amersham International plc, Amersham, UK); and *Sma* I (New England Biolabs, Beverly, MA, USA). Calf Intestinal Phosphatase (CIP) (New England Biolabs) was used to treat vector digests prior to ligation as per the manufacturers instructions. Ligations were performed at
15 temperatures between 12°C and 15°C overnight using T4 DNA ligase (Boehringer Mannheim GmbH) as per the manufacturers instructions. Prior to use in transformations, ligation mixtures were ethanol precipitated with 1 µl glycogen (Boehringer Mannheim GmbH) and resuspended in 10 µl Milli-Q water.

Unless otherwise stated, gel electrophoresis was performed using 1% agarose
20 (Sigma) gels prepared and run with Tris-acetate EDTA (TAE) buffer (per litre: 4.84 g Tris base (Serva), 1.142 ml glacial acetic acid (Rhône-Poulenc Chemicals Ltd., Bristol, UK), and 0.8 ml 0.5 M ethylenediaminetetra-acetate (BDH Laboratory Supplies, Poole, UK) (EDTA) at 75 - 100 V. Electrophoresis was performed using a Pharmacia Electrophoresis Constant Power Supply ECPS 2000/300 (Pharmacia Fine
25 Chemicals AB, Uppsala, Sweden), and gel electrophoresis apparatus including a range of submarine gel tanks: 20 cm x 24 cm Model H4 (Bethesda Research Laboratories, Gaithersburg, MD, USA), 11 cm x 14 cm HORIZON 11*14 (GIBCO BRL), 8 cm x 6 cm minigel tank (Bio-rad).

30 ***E. coli* DH5αF' electro-transformation.**

Unless otherwise stated, preparation of electro-competent *E. coli* DH5αF' cells and electro-transformation of electro-competent *E. coli* DH5αF' cells was performed as previously described (Dower (1988)). *E. coli* DH5αF' electro-transformations were performed with a Biotechnologies and Experimental Research Inc. (BTX) BTX® *E.*

coli TransPorator™ (BTX, SanDiego, CA, USA), a Pharmacia LKB 2197 Power Supply (Pharmacia LKB, Broma, Sweden), and 0.1 cm electrode gap Gene Pulser™ Cuvettes (Bio-rad Laboratories, Hercules, CA, USA). 40 µl aliquots of *E. coli* DH5αF' electro-competent cells were maintained at -70°C until required. Following electro-
5 poration, 1 ml of 2xYT broth was immediately added to the transformation mixture and the cells resuspended and transferred to a glass vial. Resuspended cells were incubated at 37°C with shaking at 200 rpm for 1 hour to enable the plasmid encoded antibiotic resistance genes to be expressed. Dilutions of the mixture were spread plated on appropriate antibiotic-containing media and incubated at 37°C
10 overnight.

Characterisation of *E. coli* DH5αF' transformants carrying recombinant pBluescript® II SK(+) phagemid vectors.

Colonies growing on LBA+Ap were patched with a sterile toothpick onto LBA+Ap
15 screening plates spread with 4 µl of 200 mg/ml Isopropyl-b-D-thiogalactoside (IPTG) (Boehringer Mannheim GmbH) and 40 µl of 20 mg/ml 5'-Bromo-4-chloro-3-indoyl-b-D-galactopyranoside (X-gal) (Boehringer Mannheim GmbH). After overnight incubation *E. coli* DH5αF' transformants containing Bluescript® II SK(+) phagemid
20 vectors (Stratagene) (Alting-Mees *et al*, 1989; Short *et al*, 1988) with inserts were identified as white patches amongst a background of blue patches. A small amount of culture was picked from each white patch with a toothpick and resuspended in 25 µl of cracking solution (In one ml: 835 µl MQ water, 100 µl glycerol (BDH), 25 µl 20% Sodium Dodecyl Sulphate (SDS) (BDH), 25 µl 2 M NaOH (BDH), 10 µl 0.5 M EDTA (BDH) and 5 µl 2% bromocresol green (J.T. Baker Co., Phillipsburg, NJ, USA))
25 and incubated at 65°C for 30 minutes. After incubation each sample was carefully loaded into dry wells in an agarose gel and electrophoresed at 40 V for approximately 15 minutes until each sample had completely entered the gel. TAE buffer was then added to cover the gel and electrophoresis continued at 75 - 100 V until completion. DNA bands were visualized by staining the gel for 10 minutes in
30 0.5 µg/ml ethidium bromide (Sigma) solution. Supercoiled plasmids were clearly visible after ethidium bromide staining. Recombinants were initially characterized by comparing their plasmid size with the plasmid size of supercoiled pBluescript® II SK(+) phagemid vector carrying no insert.

E. coli DH5 α F' transformants yielding appropriately sized plasmids were used to inoculate 2.5 ml 2xYT broth supplemented with 100 μ g/ml ampicillin. Following overnight incubation at 37°C plasmid DNA was extracted from 1.5 ml of each culture using the Quantum prep™ plasmid miniprep kit (miniprep) (Bio-rad) and the plasmid DNA eluted from the miniprep matrix in 100 μ l of MQ water according to the manufacturers instructions. The eluted DNA was stored at -20°C. The remaining culture was centrifuged and the pellet resuspended in 10% skim milk and stored at -70°C.

Those transformants carrying pBluescript® II SK(+) phagemid vector with an insert were characterized by restriction digestion of miniprep plasmid DNA. Plasmid DNA was digested with restriction enzymes chosen to linearise the plasmid. *Eco*R I was used to linearise plasmid DNA from pSB1006, pSB1291, pSB1205, and pSB1014 transformants. *Sac* I was used to linearise plasmid DNA from pSB10313 and pSB1047 transformants, *Hind* III to linearise plasmid DNA from pSB1083 transformants, and *Pst* I to linearise plasmid DNA from pSB961 and pSB981 transformants. The digested plasmid DNA was electrophoresed and the size of the plasmid determined relative to known DNA sizing standards (either *Pst* I or *Hind* III digested λ DNA (New England Biolabs)). DNA bands were visualized by staining the gel for 10 minutes in 0.5 μ g/ml ethidium bromide (Sigma) solution. The size estimate obtained for each plasmid was compared with the predicted size determined from the previously published restriction map of pDN488L (Simmonds *et al* (1997)).

Characterisation of *E. coli* DH5 α F' transformants carrying recombinant pVA838 vectors.

E. coli DH5 α F' colonies visible on LBA+Em250 after 12 - 16 hours incubation were streaked onto LBA+Em500 and LBA+Cm plates and incubated overnight at 37°C. Transformants able to grow overnight on LBA+Em500 but not on LBA+Cm were initially characterized as previously described (Characterisation of *E. coli* DH5 α F' transformants carrying recombinant pBluescript® II SK(+) phagemid vectors) and the size of their supercoiled plasmids compared with the size of supercoiled pVA838 (Macrina *et al* (1982)).

E. coli DH5 α F' isolates identified as carrying plasmids of the appropriate size were grown overnight at 37°C in 5 ml 2xYT broth supplemented with 500 μ g/ml Em. Plasmid DNA was extracted from 3 ml of each culture using the Quantum prep™ plasmid miniprep kit (Bio-rad) and the plasmid DNA eluted from the miniprep matrix in 100 μ l of MQ water according to the manufacturers instructions. The eluted DNA was stored at -20°C. The remaining culture was centrifuged and the pellet resuspended in 10% skim milk and stored at -70°C.

Transformants carrying pVA838 vector with an insert were characterized by restriction digestion of miniprep plasmid DNA essentially as described previously (Characterisation of *E. coli* DH5 α F' transformants carrying recombinant pBluescript® II SK(+) phagemid vectors). *Eco* R I was used to linearise plasmid DNA from pSB1847 transformants whereas *Eco* R I digestion of plasmid DNA from pSB1311 transformants yielded two fragments (ie. 6.8 kb insert and 9.2 kb vector).

Construction of subclones using pBluescript® II SK(+) phagemid vector.

Plasmids were constructed using a subcloning strategy based on the previously published restriction map of pDN488L (Simmonds *et al* (1997)). The cloning of pDN488L, pDN2.2, and pDN0.8 has been previously described. Unless otherwise stated the following method was used to construct all pBluescript® II SK(+) phagemid vector subclones.

At least 1 μ g pBluescript® II SK(+) phagemid vector miniprep DNA was digested with the appropriate restriction enzyme(s), treated with CIP and electrophoresed. Unless otherwise stated, at least 1 μ g of the appropriate parent plasmid miniprep DNA was digested with the appropriate restriction enzyme(s), treated with CIP and electrophoresed. Bands corresponding to the 2.9 kb linearised pBluescript® II SK(+) phagemid vector, and the desired insert fragment (Table 1) were extracted from the gel using a Prep-A-Gene™ DNA purification kit (Bio-rad), eluted with 30 μ l MQ water according to the manufacturers instructions and ligated. Following ligation of the vector and insert, electro-competent *E. coli* DH5 α F' were transformed as previously described (*E. coli* electro-transformation) and transformants isolated and characterized as previously described (Characterisation of *E. coli* transformants carrying recombinant pBluescript® II SK(+) phagemid vectors).

An alternative method was used to construct pSB1006, pSB1014, and pSB1025. A restriction enzyme was chosen that cut once within the 6.8 kb insert of pDN488L and once within the pDN488L multi-cloning site (MCS). Restriction digestion produced two fragments, one corresponded to linearised pBluescript® II SK(+) phagemid vector incorporating a section of pDN488L, and the other corresponded to the remaining region of pDN488L and a short segment of the MCS. The digest was electrophoresed and the band corresponding to linearised pBluescript® II SK(+) phagemid vector incorporating pDN488L DNA was extracted from the gel using a Prep-A-Gene™ DNA purification kit (Bio-rad), eluted with 30 µl MQ water according to the manufacturers instructions and self-ligated. Following self-ligation, electro-competent *E. coli* DH5αF' were transformed as previously described (*E. coli* electro-transformation) and transformants isolated and characterized as previously described (Characterisation of *E. coli* transformants carrying pBluescript® II SK(+) phagemid vectors). pSB1083 was constructed similarly, differing in that the parent plasmid was pSB1014. pSB1047 was constructed similarly, differing in that the parent plasmid was pSB1006 and that two enzymes with unique but compatible restriction sites were used to digest pSB1006.

pSB961 was pBluescript® II SK(+) phagemid vector incorporating the 0.7 kb *Eco* RV - *Pst* I fragment of pDN2.2.

pSB981 was pBluescript® II SK(+) phagemid vector incorporating the 1.5 kb *Eco* RV - *Pst* I fragment of pDN2.2.

pSB1006 was pBluescript® II SK(+) phagemid vector incorporating the 3.7 kb *Cla* I - *Eco*R I fragment of pDN488L. A *Cla* I digestion of pDN488L was electrophoresed and the 6.6 kb band was extracted from the gel and self-ligated as described previously (Construction of clones using pBluescript® II SK(+) phagemid vectors).

pSB1014 was pBluescript® II SK(+) phagemid vector incorporating the 3.1 kb *Hind* III - *Eco*R I fragment of pDN488L. A *Hind* III digestion of pDN488L was electrophoresed and the 6.0 kb band extracted from the gel and self-ligated as

described previously (Construction of clones using pBluescript® II SK(+) phagemid vectors).

5 pSB1025 was pBluescript® II SK(+) phagemid vector incorporating the 3.4 kb *Eco* RV - *Eco*R I fragment of pDN488L. An *Eco* RV digestion of pDN488L was electrophoresed and the 6.3 kb band extracted from the gel and self-ligated as described previously (Construction of clones using pBluescript® II SK(+) phagemid vectors).

10 pSB1083 was pBluescript® II SK(+) phagemid vector incorporating the 2.3 kb *Hind* III - *Xba* I fragment of pSB1014. A *Xba* I digestion of pSB1014 was electrophoresed and the 5.2 kb band extracted from the gel and self-ligated as described previously (Construction of clones using pBluescript® II SK(+) phagemid vectors).

15 pSB10313 was pBluescript® II SK(+) phagemid vector incorporating the 0.8 kb *Xba* I - *Eco*R I fragment of pSB1014.

20 pSB1047 was pBluescript® II SK(+) phagemid vector incorporating the 0.2 kb *Cla* I - *Eco* RV fragment of pSB1006. An *Eco* RV/*Sma* I digestion of pSB1006 was electrophoresed and the 3.1 kb band extracted from the gel and self-ligated as described previously (Construction of clones using pBluescript® II SK(+) phagemid vectors).

25 pSB1097 was pBluescript® II SK(+) phagemid vector incorporating the 0.3 kb *Hind* III - *Eco*R I fragment of pSB1025.

pSB1291 was pBluescript® II SK(+) phagemid vector incorporating the 4.0 kb *Pst* I - *Eco*R I fragment of pDN488L.

30 **Construction of clones using pVA838 vector.**

The following procedure was used to construct pSB1311 in *E. coli* DH5 α F'. pVA838 miniprep DNA (at least 1 μ g) was digested with *Eco*R I, treated with CIP and electrophoresed. pDN488L miniprep DNA (at least 1 μ g) was digested with *Eco*R I,

treated with CIP and electrophoresed. Bands corresponding to the 9.2 kb *EcoR* I digested pVA838 vector and the 6.8 kb *EcoR* I digested pDN488L insert were extracted from the gel using the Prep-A-Gene™ DNA purification kit (Bio-rad) and eluted with 30 µl MQ water according to the manufacturers instructions. Following

5 ligation of the vector and insert, electro-competent *E. coli* DH5αF' were transformed as previously described (*E. coli* electro-transformation) and transformants isolated and characterized as previously described (Characterisation of *E. coli* transformants carrying recombinant pVA838 vectors).

10 The following procedure was used to construct pSB1847 in *E. coli* DH5αF'. pVA838 miniprep DNA (at least 1 µg) was digested with *EcoR* I and *Pvu* II, treated with CIP and electrophoresed. pSB1291 miniprep DNA (at least 1 µg) was digested with *EcoR* I and *Sma* I and electrophoresed. Bands corresponding to the 8.9 kb *EcoR* I/*Pvu* II digested pVA838 vector and the 4 kb *EcoR*I/*Sma* I pSB1291 insert were extracted

15 using the Bio-rad Gel Extraction Kit (Bio-rad) and eluted with 30 µl MQ water according to the manufacturers instructions. Following ligation of the vector and insert, electrocompetent *E. coli* DH5αF' were transformed as previously described (*E. coli* electro-transformation) and transformants isolated and characterized as previously described (Characterisation of *E. coli* transformants carrying recombinant

20 pVA838 vectors).

Transformation of *S. gordonii* DL1 with pSB1311 and pSB1847.

S. gordonii DL1 was freshly subcultured on CAB prior to each transformation. 50 µl of an overnight culture of *S. gordonii* DL1 in BHS broth was used to inoculate 5 ml

25 of pre-warmed BHS broth and the culture incubated (with a loosened cap) at 37°C in 5% CO₂ in air for 3 hours. 50 µl of this was used to inoculate 5 ml of pre-warmed BHS broth and the culture incubated (with a loosened cap) at 37°C in 5% CO₂ in air for a further one hour. After one hour the culture was dispensed in 0.8 ml volumes into glass vials and mixed with 10 - 50 µl (containing a minimum of 1 µg of DNA) of

30 pSB1311 and pSB1847 miniprep DNA obtained from *E. coli* DH5αF' (pSB1311) and (pSB1847). Vials containing *S. gordonii* DL1 cells and pVA838 with no insert or *S.gordonii* DL1 cells and no DNA were included in each experiment as positive and negative controls respectively. Transformation mixtures were incubated for 3 - 4

hours at 37°C in 5% CO₂ in air before dilutions of each mixture were spread plated on CAB+Em and the plates incubated for 24 hours at 37°C in 5% CO₂ in air.

After incubation colonies were picked from the transformation plates, streaked onto CAB+Em and incubated overnight at 37°C in 5% CO₂ in air. Plasmid DNA was extracted from each isolate as previously described (Vriesema *et al*, 1996) and resuspended in 30 µl MQ water. *S. gordonii* DL1 plasmid DNA obtained in this way was characterized by restriction analysis as previously described (Characterisation of *E. coli* DH5αF' transformants carrying recombinant pVA838 vectors). Plasmid DNA extracted from *S. gordonii* DL1 (pSB1311) and (pSB1847) transformants was similarly compared with plasmid DNA extracted from *E. coli* DH5αF' (pSB1311) and (pSB1847) transformants respectively. The *E. coli* DH5αF' plasmid DNA used for comparison with the *S. gordonii* DL1 plasmid DNA originated from the same miniprep sample used in the respective *S. gordonii* DL1 transformation. Transformants were stored in 10% skim milk at -70°C.

iii) Phenotypic characterization of DL1 transformants.

Testing for BLIS production by deferred antagonism.

BLIS production was assessed using the deferred antagonism procedure (Tagg & Bannister (1979)). Briefly, a 1-cm wide streak of the test strain was inoculated diametrically across the surface of CAB plates using a cotton swab heavily charged with cells from a freshly grown THB culture. The inoculated plates were incubated at 37°C for 18 hour in air plus 5% CO₂ after which the visible growth was removed by scraping with the edge of a glass slide. The surface of the medium was sterilized by exposure to chloroform vapour for 30 minutes, aired for 30 minutes and the nine standard indicator strains (I1, *Micrococcus luteus*; I2, *S. pyogenes*; I3, *S. anginosus*; I4, *S. uberis*; I5, *S. pyogenes*; I6, *Lactococcus lactis* subsp. *lactis*; I7, *S. pyogenes*; I8, *S. pyogenes* and I9, *S. equisimilis*) (Tagg *et al*, 1979) inoculated from 18 hour THB cultures across the line of the original producer strain with use of cotton swabs. After incubation for 18 hours in 5% CO₂ at 37 °C the extent of inhibition of each indicator strain was recorded as: '-' for no inhibition and '+' if the zone was wider than each edge of the producer streak.

Testing for BLIS production by the surface spot method.

BLIS activity in liquid samples was quantitated using the surface spot method (SSM) described by Jack (1991). Briefly, a 20 µl droplet of the sample to be tested was spotted out on the surface of a CAB plate and left to soak into the agar plate. The plate surface was then sterilized by exposure to chloroform vapour for 30 minutes, aired for 30 minutes and standard indicator I2 (overnight culture in THB broth) swabbed evenly onto the surface of the plate. Following overnight incubation at 37°C for 18 hours in air plus 5% CO₂, the presence of inhibitory activity was visualized as a circular zone of inhibition in the I2 lawn at the site of droplet deposition. The titre of inhibitory activity in the samples were determined by making doubling dilutions of the test samples and plating out 20 ml drops of each dilution. The reciprocal of the highest doubling dilution at which inhibitory action was observed is given as the titre.

Testing for Zoocin A production.

S. gordonii DL1, *S. gordonii* DL1 (pVA838) and *S. gordonii* DL1 (pSB1311) and (pSB1847) were tested for zoocin A production by the deferred antagonism method.

Testing for sensitivity to Zoocin A.

S. gordonii DL1, *S. gordonii* DL1 (pVA838) and *S. gordonii* DL1 (pSB1311) and (pSB1847) were tested for sensitivity to zoocin A by both a modification of the deferred antagonism method, and a modification of the SSM. In the modified deferred antagonism method, the zoocin A producer strain, *S. equi* subsp. *zooepidemicus* 4881 was used as the test strain and *S. gordonii* DL1, *S. gordonii* DL1 (pVA838) and *S. gordonii* DL1 (pSB1311) and (pSB1847), standard indicators I1 and I2 and *S. equi* subsp. *zooepidemicus* 4881 used as the indicator strains. In the modified SSM, a partially purified preparation of zoocin A was diluted two-fold and 20 ml drops spotted onto the surface of CAB plates. The presence of inhibitory activity was visualized by swabbing onto the surface of each plate a lawn of either *S. gordonii* DL1, *S. gordonii* DL1 (pVA838) and *S. gordonii* DL1 (pSB1311) or (pSB1847), standard indicator I1 or I2 or *S. equi* subsp. *zooepidemicus* 4881.

iv) Sequencing the regions flanking zooA.**Subcloning and primer selection.**

Plasmid DNA used for double stranded DNA sequencing was obtained from *E. coli* DH5 α F' or *E. coli* XL1 blue pBluescript[®] II SK(+) phagemid vector subclones by miniprep. *E. coli* DH5 α F' and XL1 blue pBluescript[®] II SK(+) phagemid vector subclones have been previously described (See Figure 2 and section; Construction of subclones using pBluescript[®] II SK(+) phagemid vectors).

Table 2 contains a description of the primers used in this study. Universal M13 forward and reverse primers were synthesized by the Oligonucleotide Unit (Department of Biochemistry, University of Otago, Dunedin, NZ) and all other primers were synthesized by GIBCO BRL Custom Primers (GIBCO BRL). Universal M13 forward and reverse primers were used in sequencing reactions with pDN0.8, pSB961, pSB981, pSB1006, pSB1025, pSB10313, pSB1047, pSB1083 and pSB1291 plasmid DNA. SB108.3F2 and SB108.3R2 primers were designed from the sequence data obtained from sequencing pSB1083 using universal M13 forward and reverse primers respectively. Primers SB108.3F2 and SB108.3R2 were used in sequencing reactions with pSB1083 plasmid DNA. 6.8kbcontig1 to 6.8kbcontig12 primers were designed from contiguous sequence data obtained from sequencing pDN0.8, pSB961, pSB981, pSB1006, pSB1025, pSB10313, pSB1047, pSB1083 and pSB1291 using universal M13 forward, universal M13 reverse, SB108.3F2 and SB108.3R2 primers. 6.8kbcontig1 - 6.8kbcontig12 primers were used in sequencing reactions with pDN488L plasmid DNA. ZooA SBD primer 1 was designed from the previously reported zooA sequence (Simmonds *et al* (1997)). ZooA SBD primer 1 was used in sequencing reactions with pSB981 plasmid DNA. Sequencing reactions were performed by the Centre for Gene Research (University of Otago, Dunedin, NZ) using an Applied Biosystems (ABI) 373 Version 3.0 DNA sequencer and the manufacturers' procedures and specifications.

Sequence analysis.

DNA sequence analysis was performed using an series 6100/66 Power Macintosh Apple computer. The sequence chromatographs were viewed and trimmed using the SeqEd (ABI) application. DNA sequences were compiled and a contiguous sequence was constructed using the DNASTar Seqman application. Open reading frames and putative amino acid sequences were determined using the DNASTar EditSeq application and visualized using either the DNASTar MapDraw or GeneJockey

(Biosoft, Cambridge, England) applications. DNA and amino acid sequence homology searches were performed using the non-redundant protein and nucleotide databases and the gapped basic local alignment search tool (BLAST) program of the National Centre for Biotechnology Information (NCBI) (NCBI, Bethesda, MD, USA).
5 Sequence alignments and sequence similarity calculations were performed using the DNASTar Megalign application.

Results and Technical Discussion

10 Transformation of *E. coli* DH5 α F' and characterization of transformants.

E. coli DH5 α F' were transformed by electro-poration with Bluescript[®] II SK(+) phagemid vector with a transformation efficiency of approximately 10⁶ transformants per μ g plasmid DNA. Transformation efficiency for the electro-transformations of pSB1006, pSB1014, pSB1025, pSB10313, pSB1083, and
15 pSB1097 were less than 20 transformants per μ g plasmid DNA. All other recombinant Bluescript[®] II SK(+) phagemid vectors gave transformation efficiencies of between 10³ - 10⁴ transformants per μ g plasmid DNA. 2 - 50% of *E. coli* DH5 α F' pBluescript[®] II SK(+) phagemid vector transformants screened on LBA+Ap containing IPTG and X-gal produced white colonies. 5 - 100% of white
20 transformants were initially characterized as containing the predicted recombinant pBluescript[®] II SK(+) phagemid vector. All pBluescript[®] II SK(+) phagemid vectors characterized by restriction analysis yielded banding patterns consistent with those predicted by the cloning strategy. The discrepancies observed between *E. coli* DH5 α F' transformation efficiency and the number of isolates characterized as
25 possessing plasmids with inserts were considered to be the result of minor variations in miniprep preparations, restriction digestion, gel extraction, ligation, and/or electro-poration.

pBluescript[®] II SK(+) phagemid vector subclones that involved self-ligation were the
30 simplest to characterize. Although all arose from low efficiency transformations almost 100% of white colonies were shown to carry plasmids with an appropriate insert. In contrast, many of the isolates obtained from higher efficiency transformations were difficult to characterize because of the high background of blue colonies, and the lower proportion (as few as 5%) of white colonies that were

subsequently shown to possess plasmids with an appropriate insert. The high background of blue colonies most likely arose as vectors cleaved with a single restriction enzyme recircularised due to incomplete phosphatase treatment. The high proportion of white colonies that did not harbour inserts was probably related to the use of LBA+Ap containing IPTG and X-gal plates unevenly spread with IPTG or X-gal, or the use of plates not prepared on the day of transformation.

E. coli DH5 α F' were transformed by electro-poration with pVA838 with an efficiency of 10^4 - 10^5 transformants per μ g plasmid DNA. Electro-competent *E. coli* DH5 α F' were transformed with pSB1311 and pSB1847 with an efficiency of less than 10 transformants per μ g plasmid DNA. 100% of *E. coli* DH5 α F' transformants that grew overnight on LBA+Em500, but not on LBA+Cm and were characterized by restriction analysis of plasmid DNA were shown to contain the predicted recombinant pVA838 vector. *E. coli* DH5 α F' were naturally partially resistant to erythromycin and very high concentrations were required to enable selection of pVA838 transformants expressing erythromycin resistance genes. It was noted that colonies that grew rapidly (within 12 - 16 hours) on LBA+250Em transformation plates were far more likely to contain pVA838 or recombinant pVA838 than those that grew after 16 hours. Only pVA838 or recombinant pVA838 transformants were subsequently able to grow on LBA+500Em overnight.

The genetic techniques used in the production of pSB1311 and pSB1847 transformants were essentially the same as those used to produce pBluescript[®] II SK(+) phagemid vector subclones. Presumably due to the low copy of pVA838, plasmid miniprep yields were only 25% of those obtained from minipreps of pBluescript[®] II SK(+) phagemid vector subclones. Doubling the amount of culture used to 3 ml increased yields, but increasing the volume of culture beyond 3 ml did not significantly enhance yield. Quantum prep[™] uses an adaptation of the standard alkaline lysis miniprep method (Sambrook *et al* (1989)) so there is a limit to the amount of cells that can effectively be lysed without increasing the volume of lysis buffer that is added at the same time. It is most likely that inefficient ligation due to their larger size caused the low transformation efficiencies observed with pSB1311 and pSB1847.

Construction of *E. coli* DH5 α F' subclones.

All *E. coli* DH5 α F' subclones were constructed without difficulty. pVA838 has two restriction sites within the chloramphenicol resistance determinant that are suitable for shuttle cloning between *E. coli* DH5 α F' and *S. gordonii* DL1 ie. *Eco*R I and *Pvu* II.

- 5 Use of the *Eco*R I site enabled pSB1311 to be constructed without difficulty. In contrast it was more difficult to decide the best strategy to use in constructing pSB1847. Although it was possible to use the *Pvu* II restriction sites flanking the pSB1291 MCS to directly transfer the 4.0 kb insert into pVA838 cleaved with *Pvu* II, this strategy was not favoured for a number of reasons. It has been reported that
- 10 ligating fragments with two blunt termini, as opposed to one blunt and one overhanging terminus, is less efficient. Also, pSB1311 did not contain the *lac* promoter region and there was uncertainty about the effect that its inclusion into the new construct would have on the expression of *zif*. By using only streptococcal DNA to construct pSB1847 there was little doubt that any observed gene expression
- 15 was initiated from a streptococcal promoter carried on the 4.0 kb insert and that any phenotypic differences observed between *S. gordonii* DL1 (pSB1311) and (pSB1847) transformants were a consequence of the additional 2.8 kb of DNA carried by pSB1311.

20 **Transformation of *S. gordonii* DL1 and characterization of transformants.**

- Transformation of *S. gordonii* DL1 with pVA838 gave a transformation efficiency of 10^3 transformants per μ g plasmid DNA. Transformation of *S. gordonii* DL1 with pSB1311 or pSB1847 gave an efficiency of less than 10 transformants per μ g plasmid DNA. Because of the low efficiency of transformation all transformants
- 25 suspected of carrying a recombinant pVA838 plasmid were phenotypically characterized. Restriction analysis showed plasmid DNA extracted from transformed *S. gordonii* DL1 to be identical to that obtained from the respective *E. coli* DH5 α F' strain.

- 30 The low transformation efficiency obtained with pSB1311 and pSB1847, but not with pVA838 transformations of *S. gordonii* DL1 is unlikely to be due to genes carried on the respective inserts as transformants appeared normal in all respects other than their zoocin A resistant zoocin A producer phenotype. pVA838 in *S. gordonii* DL1 was very stable, and pSB1311 and pSB1847 were also able to be

maintained without antibiotic selection. It is more likely that the larger size of pSB1311 and pSB1847 made DNA uptake by competent *S. gordonii* DL1 cells less efficient.

5 **Phenotypic characterization of strains.**

The results of the testing of strains for production of and sensitivity to zoocin A by deferred antagonism are given in Table 3. That the inhibitory profile produced by *S. equi* subsp. *zooepidemicus* 4881 was the same as that produced by *S. gordonii* DL1 carrying pSB1311 but not *S. gordonii* DL1 carrying pSB1847 confirming that *zooA* is
10 essential for zoocin A production. A partially purified preparation of zoocin A produced endpoint titres of 2048, 128, 128, 0, 0, 0 and 0 when tested by SSM against standard indicator I2, *S. gordonii* DL1, *S. gordonii* DL1 (pVA838), *S. gordonii* DL1 (pSB1847), *S. gordonii* DL1 (pSB1311), standard indicator I1 and *S. equi* subsp. *zooepidemicus* 4881 respectively.

15

A summary of the results of the phenotypic testing of *S. gordonii* DL1 transformants is given in Table 4.

Sequence data and sequence analysis.

20 The subcloning strategy used enabled much of the 6.8 kb *EcoR* I fragment sequence to be established by sequencing from both ends of each subclone from M13 universal forward and reverse primers. Three internal primers were required to complete the single stranded contiguous sequence of the entire 6.8 kb fragment. Fragments carried by pSB1083 and pSB981 were too large to be sequenced
25 completely with the M13 universal primers, consequently SB1083R2 and SB1083F2 primers were designed to enable sequencing of the remaining undetermined region within the 2.3 kb pSB1083 insert. SBD primer 1 was used to complete the sequencing of pSB981. To obtain a double stranded contiguous sequence the 6.8kbcontig1 - 12 primers were designed and used in sequencing reactions with
30 pDN488L.

The nucleotide sequence of the 6.8 kb *EcoR* I fragment is given in Figure 3 and the identified open reading frames (ORF) are given in Figure 2. Sequence analysis indicated the presence of an ORF encoding a 411 amino acid protein (including the
35 "stop" residue) which we have called *zif*. (zoocin A immunity factor). That *zif* is

essential for zoocin A immunity is supported by the observation that zoocin A inhibited *S. gordonii* DL1 and *S. gordonii* DL1 pVA838, but not *S. gordonii* DL1 carrying pSB1311 or pSB1847. *zif* is located on the 4.0 kb *EcoR* I - *Pst* I fragment of pDN488L that is common to both pSB1311 and pSB1847.

5

Three further ORFs were identified (Figure 2). ORF 1 encodes a 142 amino acid sequence with homology to the 5' region of *rgg* which regulates expression of glucosyltransferase in *S. gordonii* CH1. ORF 2 encodes a 244 amino acid sequence with homology to insertion sequence IS200 found in a range of bacteria including
10 *Clostridium perfringens*, *E. coli*, and *Yersinia pestis*. However, ORF 2 is most closely related to an IS200 sequence identified in *S. pneumoniae*. ORF 3 encodes a 394 amino acid sequence with homology to a transposase/insertion sequence also identified in *S. pneumoniae*.

Table 1. Bacterial strains and plasmids used in this study.

Species, strain, and (plasmid)	Size (kb) of		Selective antibiotic ^c	Strain and plasmid references
	Plasmid	Insert		
<i>E. coli</i>				
XL1-blue (pDN0.8) ^a	3.5	0.6	Ap100	Simmonds <i>et al</i> (1997)
XL1-blue (pDN2.2) ^a	5.1	2.2	Ap100	Simmonds <i>et al</i> (1997)
XL1-blue (pDN488L) ^a	9.7	6.8	Ap100	Simmonds <i>et al</i> (1997)
DH5aF' (pSK [®] II(+)) ^a	2.9	No insert	Ap100	Woodcock <i>et al</i> (1989); Raleigh <i>et al</i> (1989); Alting-Mees and Short (1989); Short <i>et al</i> (1988)
DH5aF' (pSB961) ^a	3.6	0.7	Ap100	herein
DH5aF' (pSB981) ^a	4.4	1.5	Ap100	herein
DH5aF' (pSB1006) ^a	6.6	3.7	Ap100	herein
DH5aF' (pSB1025) ^a	6.3	3.4	Ap100	herein
DH5aF' (pSB1014) ^a	6.0	3.1	Ap100	herein
DH5aF' (pSB10313) ^a	3.7	0.8	Ap100	herein
DH5aF' (pSB1047) ^a	3.1	0.2	Ap100	herein
DH5aF' (pSB1083) ^a	5.2	2.3	Ap100	herein
DH5aF' (pSB1097) ^a	3.2	0.3	Ap100	herein
DH5aF' (pSB1291) ^a	6.9	4.0	Ap100	herein Macrina <i>et al</i> (1982)
DH5aF' (pVA838) ^b	9.2	No insert	Cm25, Em500	herein
DH5aF' (pSB1311) ^b	16.0	6.8	Cm25, Em500	herein
DH5aF' (pSB1847) ^b	13.2	4.0	Cm25, Em500	herein
<i>S. gordonii</i>				
DL1 (pVA838) ^b	9.2	No insert	Em10	Macrina <i>et al</i> (1982)
DL1 (pSB1311) ^b	16.0	6.8	Em10	herein
DL1 (pSB1847) ^b	13.2	4	Em10	herein

a Parent vector, pBluescript[®] II SK(+) phagemid vector (Stratagene).

b Parent vector, pVA838 (kindly donated by Dr H. Jenkinson, Dept. of Oral Biology, University of Otago, Dunedin, NZ).

5 c Antibiotic abbreviations; Ap100, 100 mg/ml ampicillin; Cm25, 25 mg/ml chloramphenicol; Em500, 500 mg/ml erythromycin and Em10, 10 mg/ml erythromycin.

Table 2. Primers used in this study.

Primer		
Designation	sequence ^a	position & orientation
Universal M13 reverse	GGAAACAGCTATGACCATG	806 (+) ^b
Universal M13 forward	GTAAAACGACGGCCAGT	579 (-) ^b
SB108.3R2	TGAGTGAAGCAACTG	1214 (+) ^c
SB108.3F2	TTATGCTCCAGCACT	2680 (-) ^c
ZooA SBD primer 1	GGGTTGATAATGG	4547 (+) ^c
6.8kbcontig1	AGTCTGTAGGTTTCGTATTCT	1375 (-) ^c
6.8kbcontig2	TGTGGCTTCATTAGGTCCAA	1754 (+) ^c
6.8kbcontig3	AGTACTGTTGGACCTAATGA	1780 (-) ^c
6.8kbcontig4	TGCGGGTGCGCGACGAAGGT	2212 (-) ^c
6.8kbcontig5	TTGGGTATAACCTTCGTCGC	2184 (+) ^c
6.8kbcontig6	TTCCCAGTAATACCTAACAT	2592 (+) ^c
6.8kbcontig7	TCATAATACTCAAGTCCTTT	3024 (+) ^c
6.8kbcontig8	AATATCAAGTTCTAATACAT	3375 (+) ^c
6.8kbcontig9	TCAATCTTGCTCTGTCCTT	5050 (+) ^c
6.8kbcontig10	CGTCTTTTGAGCTACTCTGA	5231 (-) ^c
6.8kbcontig11	GGCGAATCAAAGTCTTG TAG	5910 (+) ^c
6.8kbcontig12	TTCTCGATTGCGCAGGCTAC	5945 (-) ^c

a Primer sequence is presented 5' to 3'.

5 b Primer position is given as the first nucleotide of the primer relative to the sequence of the pBluescript® II SK (+) phagemid vector as previously described (Short *et al*, 1988; Alting-Mees *et al*, 1989).

c Primer position is given as the first nucleotide of the primer relative to the sequence of the 6.8 kb fragment of pDN488L as designated in Figure 3.

Table 3. Production and sensitivity to BLIS of strains tested by deferred antagonism.

Indicator strains	Producer strain				
	4881 ^a	DL1 ^b	pVA838 ^c	pSB1311 ^d	pSB1847 ^e
I1	-	-	-	-	-
I2	+	-	-	+	-
I3	-	-	-	-	-
I4	-	-	-	-	-
I5	+	-	-	+	-
I6	-	-	-	-	-
I7	+	-	-	+	-
I8	+	-	-	+	-
I9	+	-	-	+	-
4881	-	-	-	-	-
DL1	+	-	-	+	-
pVA838	+	-	-	+	-
pSB1311	-	-	-	-	-
pSB1847	-	-	-	-	-

5 a *S. equi* subsp. *zooepidemicus* 4881.

b *S. gordonii* DL1.

c *S. gordonii* DL1 (pVA838).

d *S. gordonii* DL1 (pSB1311).

e *S. gordonii* DL1 (pSB1847).

10

Table 4. Phenotypic characterization of *S. gordonii* DL1 clones.

		Phenotype		
Strain and plasmid	Genotype ^a	Zoocin A production	Zoocin A immunity	Em resistance ^b
<i>S. gordonii</i>				
DL1	<i>zooA</i> - <i>zif</i> - Em ^S	-	-	-
DL1 (pVA838)	<i>zooA</i> - <i>zif</i> - Em ^R	-	-	+
DL1 (pSB1311)	<i>zooA</i> + <i>zif</i> + Em ^R	+	+	+
DL1 (pSB1847)	<i>zooA</i> - <i>zif</i> + Em ^R	-	+	+
<i>S. equi</i> subsp. <i>zooepidemicus</i>				
4881	<i>zooA</i> + <i>zif</i> + Em ^S	+	+	-

5 a *zooA* +/- denotes the prescence or absence of the gene encoding zoocin A, *zif* +/- denotes the prescence or absence of the gene encoding zoocin A immunity, Em^R denotes the presence of the erythromycin resistance gene located on pVA838 and Em^S indicates no erythromycin resistance gene.

10 b Denotes sensitivity or resistance to 10 µg/ml erythromycin.

The foregoing examples are illustrations of the invention. The invention may be carried out with numerous variations and modifications as will be apparent to those skilled in the art. For example, the native *zif* gene need not be used in the transformation. Deletions, insertions and substitutions relative in the *zif* gene may be used provided that the *zif*-type activity is retained. Similarly the gene may be incorporated into species other than used in Example 1. Likewise there are many variations in the way in which the invention can be used in pharmaceuticals and food products.

REFERENCES

- Alting-Mees, M.A. and J.M. Short. 1989. pBluescript II: gene mapping vectors. Nucleic Acids Res. 17: 9494.
- 5 Altschul, S.F. *et al.* 1990. J. Mol. Biol., 215:403-410.
- Bronze, M.S. and J.B. Dale. 1996. The reemergence of serious group A streptococcal infections and acute rheumatic fever. Am. J. Med. Sci. 311: 41-54.
- 10 Dower, W.J. 1988. Transformation of *E. coli* to extremely high efficiency by electroporation. Mol. Biol. Rep. 6: 3-4.
- Federal Register. 1988. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. Fed. Regist. 54: 11247-11251.
- 15 Francis, A.J., G.R. Nimmo, A. Efstratiou, V. Galanis and N. Nuttall. 1993. Investigation of milk-borne *Streptococcus zooepidemicus* infection associated with glomerulonephritis in Australia. J. Infect. 27: 317-323.
- 20 Jack, R.W. 1991. Production, purification and characterisation of the streptococcal lantibiotic streptococcin A-FF22. A Thesis. University of Otago, Dunedin, New Zealand.
- 25 Jones C.L., J.A. Ritchie, P.D. Marsh and F.J.G. van der Ouderaa. 1988. The effect of long-term use of a dentifrice containing zinc citrate and a non-ionic agent on the oral flora. J. Dent. Res. 67: 46-50.
- Loesche, W.J. 1976. Chemotherapy of dental plaque infections. Oral Sci. Rev. 9: 65-107.
- 30 Loesche, W.J., S.A. Eklund, D.F. Mehlich and B. Burt. 1989. Possible effects of medically administered antibiotics on the mutans streptococci, implications for reduction in decay. Oral Microbiol. Immunol. 4: 77-81.

- Macrina, F.L., J.A. Tobian, K.R. Jones, R.P. Evans and D.B. Clewell. 1982. A cloning vector able to replicate in *Escherichia coli* and *Streptococcus sanguis*. *Gene*. 19: 345-353.
- 5 Marsh, P.D. 1991. Dentifrices containing new agents for the control of plaque and gingivitis: microbiological aspects. *Clin. Periodontol.* 18: 462-467.
- Pearson, W.R. *et al.*, 1988. *Proc. Natl. Acad. Sci.*, 85:2444-2448.
- 10 Raleigh, E.A., K. Lech and R. Brent. 1989. Selected topics from classical bacterial genetics. In. *Current protocols in molecular biology*. eds. Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith and K. Struhl. Publishing Associates and Wiley Interscience, New York. Unit 1.4.
- 15 Rodriguez, J.M. and H.M. Dodd. 1996. Genetic determinants for the biosynthesis of nisin, a bacteriocin produced by *Lactococcus lactis*. *Microbiologia*. 12: 61-74.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. 1989. *Molecular Cloning. A Laboratory*
20 *Manual*. 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, NY, USA.
- Schofield, C.R. and J.R. Tagg. 1983. Bacteriocin-like activity of group B and group C streptococci of human and of animal origin. *J. Hyg.* 90: 7-18.
- 25 Short, J.M., J.M. Fernandez, J.A. Sorge and W.D. Huse. 1988. Lambda ZAP: a bacteriophage lambda expression vector with *in vivo* excision properties. *Nucleic Acids Res.* 16: 7583-7600.
- 30 Simmonds, R.S., J. Naidoo, C.L. Jones and J.R. Tagg. 1995. The streptococcal bacteriocin-like inhibitory substance, zoocin A, reduces the proportion of *Streptococcus mutans* in an artificial plaque. *Microb. Ecol. Health Dis.* 8: 281-292.
- 35 Simmonds, R.S., Simpson, W.J. and Tagg J.R. 1997. Cloning and sequence analysis of *zooA*, a *Streptococcus zooepidemicus* gene encoding a bacteriocin-like inhibitory

substance having a domain structure similar to that of lysostaphin. *Gene*. 189: 255-261.

5 Simmonds R.S., L. Pearson, R.C. Kennedy and J.R. Tagg. 1996. Mode of action of a lysostaphin-like bacteriolytic agent produced by *Streptococcus zooepidemicus* 4881. *Appl. Environ. Microbiol.* 62: 4536-4541.

10 Tagg, J.R. and L.V. Bannister. 1979. "Fingerprinting" b-haemolytic streptococci by their production of and sensitivity to bacteriocine-like inhibitors. *J. Med. Microbiol.* 12: 397-411.

15 Thumm, G. and F. Gotz. 1997. Studies on polysostaphin processing and characterization of the lysostaphin immunity factor (Lif) of *Staphylococcus simulans* biovar *staphylolyticus*. *Mol. Microbiol.* 23: 1251-1265.

Vriesema, A.J.M., S.A.J. Zaat and J. Dankert. 1996. A simple procedure for isolation of cloning vectors and endogenous plasmids from viridans group streptococci and *Staphylococcus aureus*. *Appl. Environ. Microbiol.* 62: 3527-3529.

20 Woodcock, D.M., P.J. Crowther, J. Doherty, S. Jefferson, E. DeCruz, M. Noyer-Weidner, S.S. Smith, M.Z. Michael and M.W. Graham. 1989. Quantitative evaluation of *Escherichia coli* host strains for tolerance to cytosine methylation in plasmid and phage recombinants. *Nucleic Acids Res.* 17: 3469-3478.

SEQUENCE LISTING**(1) GENERAL INFORMATION:**

- (i) APPLICANT: University of Otago
New Zealand Pastoral Agriculture Research Institute
Limited
- (ii) TITLE OF INVENTION: Zoocin A Immunity Factor
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESS: Russell McVeagh West-Walker
 - (B) STREET: The Todd Building, Cnr Brandon Street and Lambton Quay
 - (C) CITY: Wellington
 - (D) COUNTRY: New Zealand
- (v) COMPUTER READABLE FORM
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: Windows 95
- (vi) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: NZ 329227
 - (B) FILING DATE: 21 November 1997
- (vii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Bennett, Michael Roy
 - (B) REFERENCE/DOCKET NUMBER: 23804 MRB
- (viii) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 64 4 499 9058
 - (B) TELEFAX: 64 4 499 9306

(2) INFORMATION FOR SEQ ID NO. 1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 410 amino acids
 - (B) TYPE: amino acid
 - (C) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

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Met Lys Phe Gln Glu Ile Asp Ala Leu
5
Thr Phe Glu Lys Phe Ala Asn Thr Gln Lys Arg Arg Ser
10      15      20
Phe Glu Gln Thr Ile Glu Met Gly Asn Leu Arg Lys Ser
25      30      35
Arg Asn Phe Asp Val Lys Tyr Phe Ala Leu Phe His Leu
40      45
Glu Glu Ile Lys Val Val Ala Leu Thr Tyr Thr Gln Lys
50      55      60
Ile Phe Gly Gly Leu Asn Met Gly Ile Tyr Tyr Gly Pro
65      70
Ile Phe Ser Glu Glu Arg Tyr Leu Ala His Phe Leu Ile
75      80      85
Glu Leu Lys Lys Tyr Thr Lys Lys Asn Asn Val Leu Glu
90      95      100
Leu Asp Ile Phe Pro Tyr Asp Asp Tyr Gln Tyr Tyr Asp
105      110
Asp Glu Gly Arg Leu Ile Gln Asp Gly Asn Ile Glu Leu
115      120      125
Arg Asp Ile Phe Glu Lys Ala Gly Phe Thr Tyr Gln Gly
130      135
Asp Glu Val Gly Phe Asn Ser Glu Gln Val Thr Trp His
140      145      150
Tyr Val Lys Asp Leu Thr Asn Leu Thr Ser Glu Asn Leu
155      160      165
Leu Asn Ser Phe Ser Lys Lys Gly Arg Pro Leu Val Lys
170      175
Lys Ser Asn Thr Phe Gly Ile Lys Val Arg Lys Leu Asn
180      185      190
Lys Asp Glu Leu Gln Ile Phe Ala Asn Ile Thr Asn Asp
195      200
Thr Ala Thr Arg Arg Gly Tyr Asn Asp Lys Gly Leu Glu
205      210      215
Tyr Tyr Glu Lys Phe Phe Asp Ala Phe Lys Asp Lys Ser
220      225      230
Glu Phe Thr Ile Ala Thr Leu Asn Phe Arg Glu Tyr Leu
235      240
Gly Asn Ile Leu Asp Gly Arg His Arg Leu Glu Asn Lys
245      250      255
Ile Ser Ile Leu Gly Thr Arg Leu Asp Lys Asn Pro Asn
260      265
Ser Glu Lys Ile Lys Asn Gln Leu Arg Glu Leu Asn Ser
270      275      280
Gln Arg Glu Thr Phe Leu Ile Arg Glu Glu Glu Ala Lys
285      290      295
Ser Phe Val Lys Lys Tyr Gly Asp Glu Asp Val Val Leu
300      305
Ala Gly Ser Leu Phe Val Tyr Thr Gln Gln Glu Leu Val
310      315      320

```

Tyr	Leu	Tyr	Ser	Gly	Ser	Tyr	Val	Glu	Phe	Asn	Lys	Phe
			325					330				
Tyr	Ala	Pro	Ala	Leu	Leu	Gln	Glu	Tyr	Ala	Met	Leu	Asn
335				340					345			
Ala	Leu	Lys	Lys	Gly	Ile	Lys	Phe	Tyr	Asn	Met	Leu	Gly
	350					355					360	
Ile	Thr	Gly	Lys	Phe	Asp	Asn	Ser	Asp	Gly	Val	Leu	Cys
			365					370				
Phe	Lys	Gln	Asn	Phe	Lys	Gly	Tyr	Ile	Val	Arg	Lys	Phe
375					380					385		
Ser	Asn	Phe	Ile	Tyr	Tyr	Pro	Asn	Pro	Arg	Lys	Leu	Lys
		390				395						
Val	Ile	Gln	Leu	Ile	Lys	Ser	Ile	Leu	Arg	Arg		
400				405					410			

(2) INFORMATION FOR SEQ ID NO. 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1230 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2:

ATGAAATTTTC AAGAAATCGA TGCACCTACT TTTGAAAAAT TTGCAAATAC	50
TCAGAAAAGA CGTTCTTTTG AGCAAACCAT TGAAATGGGA AATTTAAGAA	100
AGAGTCGAAA TTTTGATGTT AAATATTTTG CTCTTTTTC A TTTGGAGGAA	150
ATAAAGGTTG TCGCACTTAC ATATACCCAA AAAATATTTG GTGGCTTGAA	200
TATGGGTATT TATTATGGAC CTATTTTCTAG TGAAGAAAGA TATCTTGCAC	250
ATTTTTTTGAT TGAATTAAAA AAATATACGA AAAAAATAA TGTATTAGAA	300
CTTGATATTT TTCCATATGA TGATTATCAA TATTATGATG ATGAAGGTAG	350
GTTAATTCAA GATGGTAATA TTGAATTAAG AGATATTTTT GAAAAAGCTG	400
GTTTTACATA TCAGGGGGAT GAAGTTGGTT TTAATAGTGA GCAAGTAACT	450
TGGCATTATG TTAAAGATTT AACTAATCTT ACATCAGAAA ATCTACTAAA	500
TTCATTTTCA AAAAAAGGAC GTCCGTTAGT AAAAAATCT AATACTTTTG	550

GAATAAAAGT TAGAAAGCTT AATAAAGATG AACTTCAAAT ATTTGCAAAT	600
ATAACAAATG ATACAGCCAC TCGTCGAGGT TATAATGACA AAGGACTTGA	650
GTATTATGAA AAATTTTTCG ATGCATTTAA AGATAAGTCA GAATTTACTA	700
TTGCAACTTT GAATTTCCGT GAGTATTTAG GCAATATATT GGATGGTCGA	750
CATAGGCTTG AGAATAAAAT TTCAATTTTA GGCCTAGGT TAGATAAAAA	800
TCCAAACTCT GAAAAAATAA AAAATCAACT TAGAGAGTTA AATAGTCAAC	850
GAGAAACATT TTTAATTAGA GAAGAAGAAG CGAAATCTTT TGTTAAGAAG	900
TATGGTGATG AGGATGTCGT TCTTGCGGGA AGCCTTTTTG TATATACTCA	950
GCAAGAATTA GTATATCTTT ATTCAGGCTC ATATGTGGAG TTTAACAAGT	1000
TTTATGCTCC AGCACTTTTA CAAGAATATG CTATGTTAAA TGCATTAAAA	1050
AAAGGAATAA AATTTTATAA TATGTTAGGT ATTACTGGGA AATTTGATAA	1100
TTCAGATGGT GTTCTATGTT TTAAACAGAA CTTTAAGGGG TATATAGTTC	1150
GTAAGTTTTT AAATTTTATT TACTACCCAA ACCCTAGAAA ATTAAAAGTT	1200
ATACAACTAA TTAAAAGCAT TTTGAGAAGG	1230

(2) INFORMATION FOR SEQ ID NO. 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 285 amino acids

(B) TYPE: amino acid

(C) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 3:

								Met	Lys	Arg	Ile	Phe	Phe
												5	
Ala	Phe	Leu	Ser	Leu	Cys	Leu	Phe	Ile	Phe	Gly	Thr	Gln	
			10					15					
Thr	Val	Ser	Ala	Ala	Thr	Tyr	Thr	Arg	Pro	Leu	Asp	Thr	
20					25					30			
Gly	Asn	Ile	Thr	Thr	Gly	Phe	Asn	Gly	Tyr	Pro	Gly	His	
		35				40						45	
Val	Gly	Val	Asp	Tyr	Ala	Val	Pro	Val	Gly	Thr	Pro	Val	
			50					55					
Arg	Ala	Val	Ala	Asn	Gly	Thr	Val	Lys	Phe	Ala	Gly	Asn	
	60				65						70		
Gly	Ala	Asn	His	Pro	Trp	Met	Leu	Trp	Met	Ala	Gly	Asn	
		75				80							
Cys	Val	Leu	Ile	Gln	His	Ala	Asp	Gly	Met	His	Thr	Gly	
85				90				95					
Tyr	Ala	His	Leu	Ser	Lys	Ile	Ser	Val	Ser	Thr	Asp	Ser	
	100				105							110	
Thr	Val	Lys	Gln	Gly	Gln	Ile	Ile	Gly	Tyr	Thr	Gly	Ala	
			115					120					
Thr	Gly	Gln	Val	Thr	Gly	Pro	His	Leu	His	Phe	Glu	Met	
	125				130						135		
Leu	Pro	Ala	Asn	Pro	Asn	Trp	Gln	Asn	Gly	Phe	Ser	Gly	
		140				145							
Arg	Ile	Asp	Pro	Thr	Gly	Tyr	Ile	Ala	Asn	Ala	Pro	Val	
150				155				160					
Phe	Asn	Gly	Thr	Thr	Pro	Thr	Glu	Pro	Thr	Thr	Pro	Thr	
	165				170							175	
Thr	Asn	Leu	Lys	Ile	Tyr	Lys	Val	Asp	Asp	Leu	Gln	Lys	
		180				185							
Ile	Asn	Gly	Ile	Trp	Gln	Val	Arg	Asn	Asn	Ile	Leu	Val	
	190			195							200		
Pro	Thr	Asp	Phe	Thr	Trp	Val	Asp	Asn	Gly	Ile	Ala	Ala	
		205				210							
Asp	Asp	Val	Ile	Glu	Val	Thr	Ser	Asn	Gly	Thr	Arg	Thr	
215				220				225					
Ser	Asp	Gln	Val	Leu	Gln	Lys	Gly	Gly	Tyr	Phe	Val	Ile	
	230				235						240		
Asn	Pro	Asn	Asn	Val	Lys	Ser	Val	Gly	Thr	Pro	Met	Lys	
		245				250							
Gly	Ser	Gly	Gly	Leu	Ser	Trp	Ala	Gln	Val	Asn	Phe	Thr	
	255			260							265		
Thr	Gly	Gly	Asn	Val	Trp	Leu	Asn	Thr	Thr	Ser	Lys	Asp	
		270				275							
Asn	Leu	Leu	Tyr	Gly	Lys								
280					285								

(2) INFORMATION FOR SEQ ID NO. 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 855 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4:

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                                ATGAAACGTA TATTTTTTGC      20
TTTCTTAAGT TTATGCTTAT TTATATTCGG AACACAAACG GTATCTGCAG      70
CTACTTATAC TCGGCCATTA GATACGGGAA ATATCACTAC AGGGTTTAAC     120
GGATACCCTG GTCATGTTGG AGTCGATTAT GCAGTACCCG TTGGAAGTCC     170
GGTTAGAGCA GTTGCAAATG GTACAGTCAA ATTTGCAGGT AATGGGGCTA     220
ATCACCCATG GATGCTTTGG ATGGCTGGAA ACTGTGTTCT AATTCAACAT     270
GCTGACGGGA TGCATACTGG ATATGCACAC TTATCAAAAA TTTCAGTTAG     320
CACAGATAGT ACAGTTAAAC AAGGACAAAT CATAGGTTAT ACTGGTGCCA     370
CCGGCCAAGT TACCGGTCCA CATTTGCATT TTGAAATGTT GCCAGCAAAT     420
CCTAACTGGC AAAATGGTTT TTCTGGAAGA ATAGATCCAA CCGGATACAT     470
CGCTAATGCC CCTGTATTTA ATGGAACAAC ACCTACAGAA CCTACTACTC     520
CTACAACAAA TTTAAAAATC TATAAAGTTG ATGATTTACA AAAAATTAAT     570
GGTATTTGGC AAGTAAGAAA TAACATACTT GTACCAACTG ATTTACATG      620
GGTTGATAAT GGAATTGCAG CAGATGATGT AATTGAAGTA ACTAGCAATG      670
GAACAAGAAC CTCTGACCAA GTTCTTCAAA AAGGTGGTTA TTTTGTGTCATC     720
AATCCTAATA ATGTTAAAAG TGTTGGAAGT CCGATGAAAG GTAGTGGTGG      770
TCTATCTTGG GCTCAAGTAA ACTTTACAAC AGGTGGAAAT GTCTGGTTAA     820
ATACTACTAG CAAAGACAAC TTACTTTACG GAAAA                      855

```


CLAIMS

1. A protein which comprises the amino acid sequence of SEQ ID NO. 1 and which is capable of protecting a host cell expressing it against zoocin A activity, or a functionally equivalent variant thereof.
- 5 2. A protein as claimed in claim 1 which has the amino acid sequence of SEQ ID NO. 1.
3. A DNA molecule which encodes a protein as claimed in claim 1.
4. A DNA molecule which comprises SEQ ID NO. 2, or a functionally equivalent variant thereof.
- 10 5. A vector which includes a DNA molecule as claimed in claim 3 or claim 4.
6. A vector as claimed in claim 5 which further includes DNA encoding a protein having zoocin A activity.
7. A vector as claimed in claim 6 wherein said protein having zoocin A activity has or includes the amino acid sequence of SEQ ID NO. 3, or a functionally
15 equivalent variant thereof.
8. A vector as claimed in claim 6 wherein said DNA encoding said protein has or includes the nucleotide sequence of SEQ ID NO. 4, or a functionally equivalent variant thereof.
9. A method of protecting an organism susceptible to the bacteriolytic activity of zoocin A against such activity which comprises the step of introducing
20 into said organism a DNA molecule according to claim 3 or claim 4.
10. A method as claimed in claim 9 wherein said DNA molecule is introduced into said organism in the form of a vector as claimed in claim 5.
11. An organism which has been rendered resistant to zoocin A activity by a
25 method as claimed in claim 9 or claim 10.
12. A method of genetically modifying a non-pathogenic organism to express a protein having zoocin A activity without said organism being itself at risk

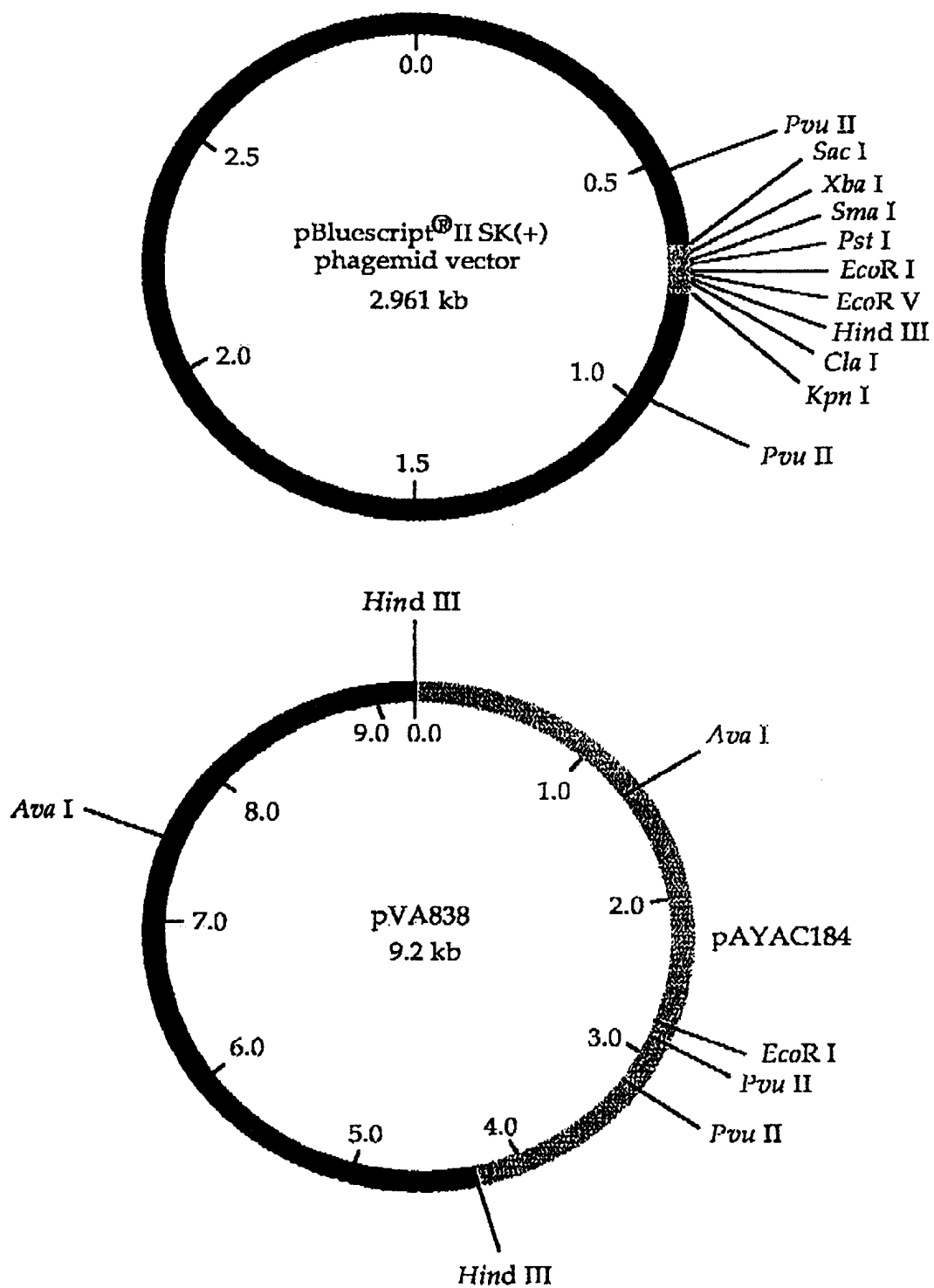
from said activity which comprises the step of introducing a DNA molecule encoding said protein into an organism as claimed in claim 11.

13. A method of genetically modifying a non-pathogenic organism to express a protein having zoocin A activity without said organism being itself at risk from said activity which comprises the step of introducing into said organism a DNA molecule encoding said protein together with a DNA molecule according to claim 3 or claim 4.
14. A method as claimed in claim 13 wherein said DNA molecules are introduced into said organism in the form of a vector as claimed in any one of claims 6 to 8.
15. A non-pathogenic organism which has been genetically modified in accordance with a method as claimed in any one of claims 12 to 14.
16. A non-pathogenic organism which is resistant against zoocin A activity and wherein said resistance is due to the presence in said organism of a DNA molecule as claimed in claim 3 or claim 4.
17. A non-pathogenic organism which expresses a protein having zoocin A activity but which is itself resistant to said activity, wherein said resistance is due to the presence in said organism of a DNA molecule as claimed in claim 3 or claim 4.
18. A non-pathogenic organism as claimed in claim 17 which is a food grade organism.
19. A non-pathogenic organism as claimed in claim 18 which is a food grade *Streptococcus*.
20. A non-pathogenic organism as claimed in claim 19 wherein the food grade *Streptococcus* is *S. gordonii*.
21. An antibacterial composition which comprises a non-pathogenic organism according to any one of claims 15 and 17 to 20.

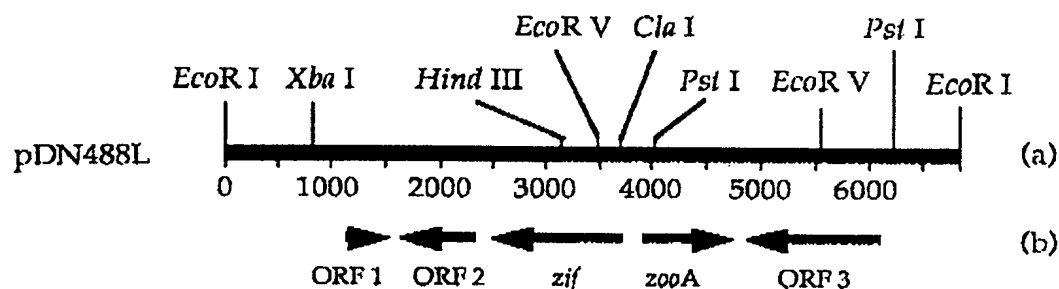
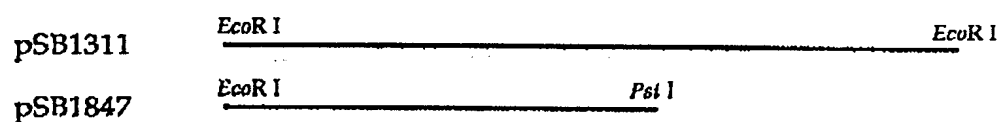
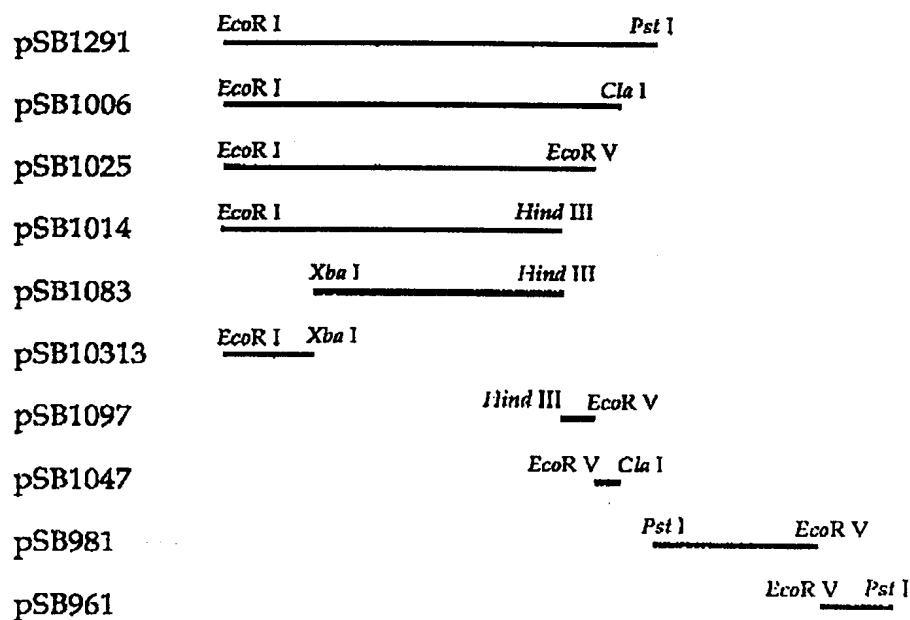
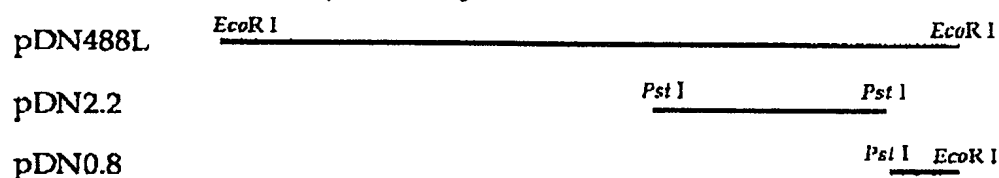
22. An antibacterial composition as claimed in claim 21 which is suitable for human ingestion.
23. An antibacterial composition as claimed in claim 21 which is suitable for ingestion by a non-human animal.
- 5 24. An antibacterial composition as claimed in claim 22 or claim 23 which is, or is part of, a foodstuff.
25. An antibacterial composition as claimed in claim 22 which is, or is part of, a nutraceutical.
- 10 26. An antibacterial composition as claimed in claim 24 or claim 25 which is or contains a dairy product.
27. An antibacterial composition as claimed in claim 22 which is, or is part of, a confectionery.
28. An antibacterial composition as claimed in claim 27 which is a wine gum or chewing gum.
- 15 29. A method of preventing or inhibiting the growth of undesirable organisms susceptible to zoocin A which comprises the step of contacting said undesirable organisms or the environment thereof with a composition as claimed in claim 21.
- 20 30. A method as claimed in claim 29 wherein said composition is administered to the oral cavity of a patient to prevent or inhibit the growth of *S. mutans*, *S. sobrinus* and/or *S. pyogenes*.
- 25 31. A method of treating or preventing *Streptococcal* sore throat or dental caries in a susceptible patient which comprises the step of orally administering to said patient a composition as claimed in claim 22.

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Figure 1. Map of pBluescript[®] II SK(+) phagemid vector and pVA838.



2/7

Figure 2. Restriction map of pDN488L showing ORFs and subclones.**pVA838 subclones.****pBluescript[®] II SK(+) subclones.****Previously described pBluescript[®] II SK(+) subclones.**

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Figure 3. DNA sequence of 6.8 kb EcoR I fragment showing *zooA* and *zlf*.

GAATTCACATCTAAGTGTGACCGACTATTTTTTTGTATTTTAGGGAA 48
 ACAAAGTCTATCTCTAGTATGCAAGCCGTTTCACGCGGAAGCAATCTGAA 98
 GAGAAGTATGTTGCCACCTGCTTATATCGTGCGGGTTCAATACAAAACG 148
 TGAATCACCGGTGCCAATACAGCTTTCCTTACCTCCTTAGCTCAGTTGGT 198
 AGAGCAGTAGACTCTTAATCTATGGGTACAGGTTTCGAGCCCTGTAGGGG 248
 GTATCATACTATACATAAAAAAGCCTTTAAATTAAGGCTTTTTTGCTTGT 298
 CTAAAGAGGATTTGTTCCACCATTGTGCCCGGAAACAATTTTTTATGATA 348
 TGATGTTAAATAAAGAATTTATTACTTTTTTAAAAAGAGAGCTAACACATG 398
 ACAATAATATAATTAACACAAGGTGATACAATCGGAGTATTTTCACCTCC 448
 ATCACCTGCGACGGAATTTGGAAAAAATGGCTTTTTTTGATTATTCGCA 498
 GCTTTAAAGTTGGACAAATAGATATTAATAATGCTCGCCTAAAAGCTGG 548
 AAAACACAAAAAGAACTCGCTAAATTAATAGGAGTTACTAAGCAAACTAA 598
 TTATTAATTACGAAAAGGGGACTACTGAACCTTCATGGGATAGACTTCAA 648
 GAGATTGCTACAGCCTTAAATGTTGATATTGATACCTTATTTCCCTACAA 698
 TATGCTAGGAGAAAAAAGAGACTTTAAGTGGATGGAGCACCTAGAGAGA 748
 CTCGAAAATAATTGGCTTTATAGCCGTATGGCCGAGGAAGAAGTATTACT 798
 TCAAAAAATTCTAGATTTTGCAATATTTCAAAATAAATTAGATAAAAACA 848
 CTCTAACAACATAAGAGCTTAATAATGAACCTAATCTTGAAGACAACAAC 898
 ACTATGTCCAAAGAAGATAAAATTTCACTCATCATATTGAAATATGAAAA 948
 AGAAATTCAAGAAAAAACTCAAAAACCTTATTGATTTATATAAAGATCAAT 998
 CAAGCAATGAATTAGATACAATAAGATTTGAATCGACTAATATATAATTC 1048
 ATATACAGAGAACGGAAGAAAAAATATTTTAAAAAAGTAGGTCATTACT 1098
 TAAATACTTGTAACAATAATATTAATAAGAATAAGTTAAATTAGCAGGA 1148
 GAGGTATATGCTAAATAAAATGGGAGAATCATTAAAAATTATGAGAAAAT 1198
 CAAGGGGAATAACTTTGAGTGAAGCAACTGGAGAAAGAAATTTTCAGAATCT 1248
 ATGCTTTCCCGTTTTGAAAATGGCCAATCCGAGATGCTTGGCTCAAAAAC 1298
 TTTTCGCTTGTTTAGATAATATTTATTTGGATATAGAAGAATATAACCTAT 1348
 TAGTTCGAGAATACGAACCTACAGACTTTTCTACACTACAAAAAACATT 1398
 CATCACTTCTACAATCCATACAATGAGATTGAGTTAGAAAAATTAGCGAA 1448
 AAAGGAAC TAGATAAAATTAATAATGATGGTCGAGAACAAATATCATAGAC 1498
 TAAATAATATATTAATCATGACCACCCGTCAAACGGGTGGTTTGAACAAA 1548
 GGCTATAAGCCACATCACCAGCCAGCGCCTAAAGACGCTGGCTTTCACT 1598
 TTGTTCAAGCCTCACCGCTTTTGACTCGTCACCAGCCTCTTAAAGAGGCG 1648
 TTCGTACTACTTACCATTATCCCTAAAGGGATCTTCATACTCTTTTACAC 1698
 TCAATTTATCAAGTGCTATATCATGTTTTTCTGTTCTTGGATATATTTT 1748
 TTAATTGTGGCTTCATTAGGTCCAACAGTACTCACATAATAGCCCTCTGC 1798
 CCAAAAATGGCGATTGCCAAATTTGTATTTGAGATTAGGGCGTTTGTCAA 1848
 ACATCATCAAAGCGCTCTTTCCCTTTCAAATATCCCATGAAACTTGACACA 1898
 CTTAATCTCGGAGGAACGCTGACTAACATGTGAACATGGTCTGGCATCAG 1948
 ATGACCTTCGATAATTTCAACACCTTTATAACGACACAAGCGTCGGAATA 1998
 TTTCTCCCAAACCTACTTCGATATTGATTAGAGATGCTTTTTTCGTCTATAC 2048
 TTAGGTGTAAAGACAATATGGTACTTGCACAACCACTTTGTATGTGATAA 2098
 ACTATGTGCCTTTTGTGCCACTTTTTCTCCTTTCACTATAACAATAGGCT 2148
 TGAACACCTTTATTGTATCGCGTTTGGAGTTTTTTTTGGGTATAACCTTCG 2198
 TCGCGCACCCGCATAGCGGGTGGTTTATTTGTCTCGCACCTTACGGAGCG 2248
 TGACGGACTTAAAGTCACATAATTAAGATAATTTCCCTTTTATATATTTCA 2298
 GAAAAATATAAAAGGAAATTGATCCTACTCTTGAACATAATTAAGTTAAAT 2348
 AATCCCATTAATAAAGGTATATGCTGTTCTGATAACATAGAAGTATTTAT 2398
 GCCTTATTTTTTAATTTTAATAGACATATGAGCTTATAGTTAAATCCCAT 2448

411 CTA CCT TCT CAA AAT GCT TTT AAT TAG TTG TAT AAC 2484
 [Stop] Arg Arg Leu Ile Ser Lys Ile Leu Gln Ile Val

FIG 3 (Cont'd)

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399	TTT	TAA	TTT	TCT	AGG	GTT	TGG	GTA	GTA	AAT	AAA	ATT	TGA	2523
	Lys	Leu	Lys	Arg	Pro	Asn	Pro	Tyr	Tyr	Ile	Phe	Asn	Ser	
386	AAA	CTT	ACG	AAC	TAT	ATA	CCC	CTT	AAA	GTT	CTG	TTT	AAA	2562
	Phe	Lys	Arg	Val	Ile	Tyr	Gly	Lys	Phe	Asn	Gln	Lys	Phe	
373	ACA	TAG	AAC	ACC	ATC	TGA	ATT	ATC	AAA	TTT	CCC	AGT	AAT	2601
	Cys	Leu	Val	Gly	Asp	Ser	Asn	Asp	Phe	Lys	Gly	Thr	Ile	
360	ACC	TAA	CAT	ATT	ATA	AAA	TTT	TAT	TCC	TTT	TTT	TAA	TGC	2640
	Gly	Leu	Met	Asn	Tyr	Phe	Lys	Ile	Gly	Lys	Lys	Leu	Ala	
347	ATT	TAA	CAT	AGC	ATA	TTC	TTG	TAA	AAG	TGC	TGG	AGC	ATA	2679
	Asn	Leu	Met	Ala	Tyr	Glu	Gln	Leu	Leu	Ala	Pro	Ala	Tyr	
334	AAA	CTT	GTT	AAA	CTC	CAC	ATA	TGA	GCC	TGA	ATA	AAG	ATA	2718
	Phe	Lys	Asn	Phe	Glu	Val	Tyr	Ser	Gly	Ser	Tyr	Ile	Tyr	
321	TAC	TAA	TTC	TTG	CTG	AGT	ATA	TAC	AAA	AAG	GCT	TCC	CGC	2757
	Val	Leu	Glu	Gln	Gln	Thr	Tyr	Val	Phe	Leu	Ser	Gly	Ala	
308	AAG	AAC	GAC	ATC	CTC	ATC	ACC	ATA	CTT	CTT	AAC	AAA	AGA	2796
	Leu	Val	Val	Asp	Glu	Asp	Gly	Tyr	Lys	Lys	Val	Phe	Ser	
295	TTT	CGC	TTC	TTC	TTC	TCT	AAT	TAA	AAA	TGT	TTC	TCG	TTG	2835
	Lys	Ala	Glu	Glu	Glu	Arg	Ile	Leu	Phe	Thr	Glu	Arg	Gln	
282	ACT	ATT	TAA	CTC	TCT	AAG	TTG	ATT	TTT	TAT	TTT	TTC	AGA	2874
	Ser	Asn	Leu	Glu	Arg	Leu	Gln	Asn	Lys	Ile	Lys	Glu	Ser	
269	GTT	TGG	ATT	TTT	ATC	TAA	CCT	AGT	GCC	TAA	AAT	TGA	AAT	2913
	Asn	Pro	Asn	Lys	Asp	Leu	Arg	Thr	Gly	Leu	Ile	Ser	Ile	
256	TTT	ATT	CTC	AAG	CCT	ATG	TCG	ACC	ATC	CAA	TAT	ATT	GCC	2952
	Lys	Asn	Glu	Leu	Arg	His	Arg	Gly	Asp	Leu	Ile	Asn	Gly	
243	TAA	ATA	CTC	ACG	GAA	ATT	CAA	AGT	TGC	AAT	AGT	AAA	TTC	2991
	Leu	Tyr	Glu	Arg	Phe	Asn	Leu	Thr	Ala	Ile	Thr	Phe	Glu	
230	TGA	CTT	ATC	TTT	AAA	TGC	ATC	GAA	AAA	TTT	TTC	ATA	ATA	3030
	Ser	Lys	Asp	Lys	Phe	Ala	Asp	Phe	Phe	Lys	Glu	Tyr	Tyr	
217	CTC	AAG	TCC	TTT	GTC	ATT	ATA	ACC	TCG	ACG	AGT	GGC	TGT	3069
	Glu	Leu	Gly	Lys	Asp	Asn	Tyr	Gly	Arg	Arg	Thr	Ala	Thr	
204	ATC	ATT	TGT	TAT	ATT	TGC	AAA	TAT	TTG	AAG	TTC	ATC	TTT	3108
	Asp	Asn	Thr	Ile	Asn	Ala	Phe	Ile	Gln	Leu	Glu	Asp	Lys	
191	ATT	AAG	CTT	TCT	AAC	TTT	TAT	TCC	AAA	AGT	ATT	AGA	TTT	3147
	Asn	Leu	Lys	Arg	Val	Lys	Ile	Gly	Phe	Thr	Asn	Ser	Lys	
178	TTT	TAC	TAA	CGG	ACG	TCC	TTT	TTT	TGA	AAA	TGA	ATT	TAG	3186
	Lys	Val	Leu	Pro	Arg	Gly	Lys	Lys	Ser	Phe	Ser	Asn	Leu	
165	TAG	ATT	TTC	TGA	TGT	AAG	ATT	AGT	TAA	ATC	TTT	AAC	ATA	3225
	Leu	Asn	Glu	Ser	Thr	Leu	Asn	Thr	Leu	Asp	Lys	Val	Tyr	

FIG 3 (Cont'd)

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152	ATG	CCA	AGT	TAC	TTG	CTC	ACT	ATT	AAA	ACC	AAC	TTC	ATC	3264	
	His	Trp	Thr	Val	Gln	Glu	Ser	Asn	Phe	Gly	Val	Glu	Asp		
139	CCC	CTG	ATA	TGT	AAA	ACC	AGC	TTT	TTC	AAA	AAT	ATC	TCT	3303	
	Gly	Gln	Tyr	Thr	Phe	Gly	Ala	Lys	Glu	Phe	Ile	Asp	Arg		
126	TAA	TTC	AAT	ATT	ACC	ATC	TTG	AAT	TAA	CCT	ACC	TTC	ATC	3342	
	Leu	Glu	Ile	Asn	Gly	Asp	Gln	Ile	Leu	Arg	Gly	Glu	Asp		
113	ATC	ATA	ATA	TTG	ATA	ATC	ATC	ATA	TGG	AAA	AAT	ATC	AAG	3381	
	Asp	Tyr	Tyr	Gln	Tyr	Asp	Asp	Tyr	Pro	Phe	Ile	Asp	Leu		
100	TTC	TAA	TAC	ATT	ATT	TTT	TTT	CGT	ATA	TTT	TTT	TAA	TTC	3420	
	Glu	Leu	Val	Asn	Asn	Lys	Lys	Thr	Tyr	Lys	Lys	Leu	Glu		
87	AAT	CAA	AAA	ATG	TGC	AAG	ATA	TCT	TTC	TTC	ACT	AAA	AAT	3459	
	Ile	Leu	Phe	His	Ala	Leu	Tyr	Arg	Glu	Glu	Ser	Phe	Ile		
74	AGG	TCC	ATA	ATA	AAT	ACC	CAT	ATT	CAA	GCC	ACC	AAA	TAT	3498	
	Pro	Gly	Tyr	Tyr	Ile	Gly	Met	Asn	Leu	Gly	Gly	Phe	Ile		
61	TTT	TTG	GGT	ATA	TGT	AAG	TGC	GAC	AAC	CTT	TAT	TTC	CTC	3537	
	Lys	Gln	Thr	Tyr	Thr	Leu	Ala	Val	Val	Lys	Ile	Glu	Glu		
48	CAA	ATG	AAA	AAG	AGC	AAA	ATA	TTT	AAC	ATC	AAA	ATT	TCG	3576	
	Leu	His	Phe	Leu	Ala	Phe	Tyr	Lys	Val	Asp	Phe	Asn	Arg		
35	ACT	CTT	TCT	TAA	ATT	TCC	CAT	TTC	AAT	GGT	TTG	CTC	AAA	3615	
	Ser	Lys	Arg	Leu	Asn	Gly	Met	Glu	Ile	Thr	Gln	Glu	Phe		
22	AGA	ACG	TCT	TTT	CTG	AGT	ATT	TGC	AAA	TTT	TTC	AAA	AGT	3654	
	Ser	Arg	Arg	Lys	Gln	Thr	Asn	Ala	Phe	Lys	Glu	Phe	Thr		
9	AAG	TGC	ATC	GAT	TTC	TTG	AAA	TTT	CAT	ATTTTATTTTCCTATA				3696	
	Leu	Ala	Asp	Ile	Glu	Gln	Phe	Lys	Met	<div>← Zif cds</div>					
	TTTCTTCCTAGTAAAATAATAACAATAAATTATAAAACGAATAAATTATAA														3747
	AAAATTTTAAGTTTCTTTTCGATTTTGTTACAATAAGTTACATTTTAAAAA														3798
	CCTTAAACTAAAAGTTGAAAATTCTATATTAATAGTTTATCATAATATTC														3849
	GAGAAGGCGCTTTCTTTTAAAGATTTATAATACTGTAATTTACTTTAGTA														3900
	AAGTTCTTTTATATGGAGGATAAAAT														3945
	<div>ZooA cds →</div> Met Lys Arg Ile Phe Phe														
7	GCT	TTC	TTA	AGT	TTA	TGC	TTA	TTT	ATA	TTC	GGA	ACA	CAA	3984	
	Ala	Phe	Leu	Ser	Leu	Cys	Leu	Phe	Ile	Phe	Gly	Thr	Gln		
20	ACG	GTA	TCT	GCA	GCT	ACT	TAT	ACT	CGG	CCA	TTA	GAT	ACG	4023	
	Thr	Val	Ser	Ala	Ala	Thr	Tyr	Thr	Arg	Pro	Leu	Asp	Thr		
33	GGA	AAT	ATC	ACT	ACA	GGG	TTT	AAC	GGA	TAC	CCT	GGT	CAT	4062	
	Gly	Asn	Ile	Thr	Thr	Gly	Phe	Asn	Gly	Tyr	Pro	Gly	His		

FIG 3 (Cont'd)

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46	GTT	GGA	GTC	GAT	TAT	GCA	GTA	CCC	GTT	GGA	ACT	CCG	GTT	4101
	Val	Gly	Val	Asp	Tyr	Ala	Val	Pro	Val	Gly	Thr	Pro	Val	
59	AGA	GCA	GTT	GCA	AAT	GGT	ACA	GTC	AAA	TTT	GCA	GGT	AAT	4140
	Arg	Ala	Val	Ala	Asn	Gly	Thr	Val	Lys	Phe	Ala	Gly	Asn	
72	GGG	GCT	AAT	CAC	CCA	TGG	ATG	CTT	TGG	ATG	GCT	GGA	AAC	4179
	Gly	Ala	Asn	His	Pro	Trp	Met	Leu	Trp	Met	Ala	Gly	Asn	
85	TGT	GTT	CTA	ATT	CAA	CAT	GCT	GAC	GGG	ATG	CAT	ACT	GGA	4218
	Cys	Val	Leu	Ile	Gln	His	Ala	Asp	Gly	Met	His	Thr	Gly	
98	TAT	GCA	CAC	TTA	TCA	AAA	ATT	TCA	GTT	AGC	ACA	GAT	AGT	4257
	Tyr	Ala	His	Leu	Ser	Lys	Ile	Ser	Val	Ser	Thr	Asp	Ser	
111	ACA	GTT	AAA	CAA	GGA	CAA	ATC	ATA	GGT	TAT	ACT	GGT	GCC	4296
	Thr	Val	Lys	Gln	Gly	Gln	Ile	Ile	Gly	Tyr	Thr	Gly	Ala	
124	ACC	GGC	CAA	GTT	ACC	GGT	CCA	CAT	TTG	CAT	TTT	GAA	ATG	4335
	Thr	Gly	Gln	Val	Thr	Gly	Pro	His	Leu	His	Phe	Glu	Met	
137	TTG	CCA	GCA	AAT	CCT	AAC	TGG	CAA	AAT	GGT	TTT	TCT	GGA	4374
	Leu	Pro	Ala	Asn	Pro	Asn	Trp	Gln	Asn	Gly	Phe	Ser	Gly	
150	AGA	ATA	GAT	CCA	ACC	GGA	TAC	ATC	GCT	AAT	GCC	CCT	GTA	4413
	Arg	Ile	Asp	Pro	Thr	Gly	Tyr	Ile	Ala	Asn	Ala	Pro	Val	
163	TTT	AAT	GGA	ACA	ACA	CCT	ACA	GAA	CCT	ACT	ACT	CCT	ACA	4452
	Phe	Asn	Gly	Thr	Thr	Pro	Thr	Glu	Pro	Thr	Thr	Pro	Thr	
176	ACA	AAT	TTA	AAA	ATC	TAT	AAA	GTT	GAT	GAT	TTA	CAA	AAA	4491
	Thr	Asn	Leu	Lys	Ile	Tyr	Lys	Val	Asp	Asp	Leu	Gln	Lys	
189	ATT	AAT	GGT	ATT	TGG	CAA	GTA	AGA	AAT	AAC	ATA	CTT	GTA	4530
	Ile	Asn	Gly	Ile	Trp	Gln	Val	Arg	Asn	Asn	Ile	Leu	Val	
202	CCA	ACT	GAT	TTC	ACA	TGG	GTT	GAT	AAT	GGA	ATT	GCA	GCA	4569
	Pro	Thr	Asp	Phe	Thr	Trp	Val	Asp	Asn	Gly	Ile	Ala	Ala	
215	GAT	GAT	GTA	ATT	GAA	GTA	ACT	AGC	AAT	GGA	ACA	AGA	ACC	4608
	Asp	Asp	Val	Ile	Glu	Val	Thr	Ser	Asn	Gly	Thr	Arg	Thr	
228	TCT	GAC	CAA	GTT	CTT	CAA	AAA	GGT	GGT	TAT	TTT	GTC	ATC	4647
	Ser	Asp	Gln	Val	Leu	Gln	Lys	Gly	Gly	Tyr	Phe	Val	Ile	
241	AAT	CCT	AAT	AAT	GTT	AAA	AGT	GTT	GGA	ACT	CCG	ATG	AAA	4686
	Asn	Pro	Asn	Asn	Val	Lys	Ser	Val	Gly	Thr	Pro	Met	Lys	
254	GGT	AGT	GGT	GGT	CTA	TCT	TGG	GCT	CAA	GTA	AAC	TTT	ACA	4725
	Gly	Ser	Gly	Gly	Leu	Ser	Trp	Ala	Gln	Val	Asn	Phe	Thr	
267	ACA	GGT	GGA	AAT	GTC	TGG	TTA	AAT	ACT	ACT	AGC	AAA	GAC	4764
	Thr	Gly	Gly	Asn	Val	Trp	Leu	Asn	Thr	Thr	Ser	Lys	Asp	
280	AAC	TTA	CTT	TAC	GGA	AAA	TAA	TTATTAA	AATTTATAGAAAT					4805
	Asn	Leu	Leu	Tyr	Gly	Lys	Stop							

FIG 3 (Cont'd)

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ATTTAATATAGACTATTAGACGTTCCCTTTTTTTGGCTCTTTGTCAACTGT	4855
AGTAGGTAGTTGACAAGCTAACATCTGGAGAGGACCAAATTGGTCTTCTC	4905
TTTTTTCATATTGATAGCGATCAAAATCCGTCTTTTAAAGTTTTCAAAGT	4955
TCCGAAATCCAAAAGCATTGCGCTTGATGACTTTGATGAGATTATTGGTA	5005
GCCTCCAGTTTAGCGTTAGAATAAGGCAAATCCAGTGCCTTCTCAATCTT	5055
GTCTCTGTCCCTTCAAAAAGGTCTTGAAAACGGTCTGAAAGAGAGGATTGC	5105
TGCTGTCTATCTGCTCCTCAATCAGGCCGAAAAAGTGGTCACCTTGCTTT	5155
TCCTGGAAGTGAAAAAGCAGAAGCTGATAAAGGTCATAATGCTTTCTAAG	5205
CTCGTCAGAGTAGCTCAAAGACGCTCGACCACCTCCTTGTTTGTCAAAT	5255
GCATGCGAAMGTGCGGGCGGTAAAAACGCTTGTCACTGAGTTTGCTGCTA	5305
TCTTGTTGAATCAGCTTCCAGTAACGTTTCAAGCCTTGTATTCGTGGCA	5355
TTTTCGATCAAAAGAGTTTCATGATTTGAGTACAGATACGGTTCATGGCAC	5405
GTCCCAAATGTTGCACAATGTGGAACGATCAAGGACAATCTGAGCGTTT	5455
GGGAAAAGCATCTTAGCCAGGTTGTAATAGGGGGTAAACATATCCATGGT	5505
GATGAGTTTAACGTCGTTTCTGACCTGCCGAGGATATCTCAGGAAGTGST	5555
TGCGAATGACAGCTTTCGTCCGTCCATCCAAAATAGCGATGATGTTGTG	5605
GTGTCAAAGTCCTGAGCAATAAAGCTCATCTTGCTCTTCTTAAAGGCATA	5655
TTCATCCCAGGACATATGTTCCGGTAAATAAGTCAAATGAGACTTGAACG	5705
TGAACTCGTTGAGCTTTCGCATGACGGTTGAGGTGGAGATGGATAGTCTA	5755
TCAGCGATAGTGGTCATGGAGACTTTCGATGAGAAGCTGGGCCACCTT	5805
CTGTTTGACGATGGTTGGTATTTGATGGTTTTTAGGGACTAGAGAAGTCT	5855
CAGCGACAGTAATTTTTCCGCAAAACTGACATTTGAAACGGCGCTTTTTG	5905
AGGCGAATCAAAGTCTTGTAGCCTGCGCAATCGAGAAAAGGGACCTTGGA	5955
TTCCCGTTGGAAGTCGTACTTGCCCATCTGACTTTGGCAGTTAGGGCAAG	6005
GTGGGGCATCGTAGTCAAGGACAGCTTTCATTCCTTATGAGTTTTTCATG	6055
TCGTGTATTTCTTTGGAGAATGTGATATGAGGGTCTTTAATTCCAGTAG	6105
TTGTGTGATAACATGTGATTGTTCCATATGAGTCTTCTAAAATGATAGTT	6155
TAGTCGCTTTTTCATTATAGGTCATATGGGACTTTTTTGATACTCATAAAG	6205
CCCTATAACCCCTGCAGTGGCCTTACCCACTACGGAAATTATACATATAT	6255
TCTTTATGCTATAATATAAGTCAAGTACAGCATTGACAAAGGAGACAAA	6305
GATTGAAAATTGTAATAATTGGCTATAGTGGTTCTGGAAAGTCGACTTTA	6355
GCAAATGTTTTAGGTCAACACTACAAATTGTGCTGTACTTCATTTAGACAA	6405
AATTCATTTTGCATCAAACCTGGCAAGAGCGAACAGTTAGTCAAATGGTCT	6455
CTGATATATCAACATTTATGTCACAAAACATTGGATTATTGAAGGTAAT	6505
TATTCAAGCTGTCTTTATGAAGAGCGTATGAGAGAGGCTGATCATTAT	6555
ATATTTTAACCTTTAATAGATTTAATTGTTTTTACCGAGCTTTTAAGCGAT	6605
ATTTAAATATAGGGGACAAACACGTCCTGATATGGCTGAAAACCTGTAAT	6655
GAAAAATTTGATGTTGAATTTATGAAATGGATTCTGTTAGACGGACGCTC	6705
AAAAAATAATTTAAATAACTATAAAACAGTTATTAAACATATCCTCATA	6755
AAATAATCGTTTTAAAAAATCAAAGCAGTTAATTCATTATATGAATTC	6804

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 98/00171

A. CLASSIFICATION OF SUBJECT MATTER																						
Int Cl ⁶ : C07K 14/315; C07H 21/04; C12N 1/21; A23L 1/00, 1/015; A01N 63/00; A61K 35/74																						
According to International Patent Classification (IPC) or to both national classification and IPC																						
B. FIELDS SEARCHED																						
Minimum documentation searched (classification system followed by classification symbols)																						
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																						
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) STN (Medline, CA): Keyword-zoocin? ANGIS (BLASTP, FASTA): sequence ID No. 1 STN subsequence search: MKFQEIDALTFEKFANTQKRRSFEQTIEMGNLRKSRNFDVKYFALFHLEEIKVVALTYTQKIFGGLNMGII YGPFISEER/SQSP																						
C. DOCUMENTS CONSIDERED TO BE RELEVANT																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
P, X	FEMS Microbiology Letters, Volume 163, 1998, S. A. Beatson et al., 'Zoocin A Immunity Factor: A Fem A-like Gene Found in a Group C <i>Streptococcus</i> ', pages 73-77. (see the whole document)	1-31																				
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input type="checkbox"/> See patent family annex																						
<p>* Special categories of cited documents:</p> <table border="0"> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier application or patent but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																			
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																			
"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 16 March 1999		Date of mailing of the international search report 24 MAR 1999																				
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929		Authorized officer <i>Marie-Anne Fam</i> MARIE-ANNE FAM Telephone No.: (02) 6283 2259																				

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ 98/00171

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Gene, Volume 189, April 1997, R. S. Simmonds et al., 'Cloning and Sequence Analysis of zooA, a <i>Streptococcus zooepidemicus</i> Gene Encoding a Bacteriocin-Like Inhibitory Substance Having a Domain Structure Similar to that of Lysostaphin', pages 255-261. (see in particular pages 256-257, 2.2 'Nucleotide sequence Analysis' and page 259, text relating to figure 2, Genbank Accession Number U50357).	3-8
A	Applied and Environmental Microbiology, Volume 62, 1996, R. S. Simmonds et al., 'Mode of Action of a Lysostaphin-like Bacteriolytic Agent Produced by <i>Streptococcus zooepidemicus</i> 4881', pages 4536-4541.	1-31